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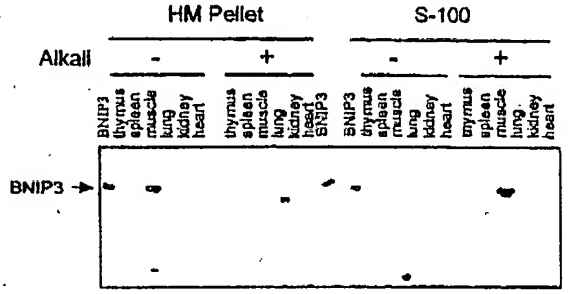
(54) Title: THE NIP3 FAMILY OF PROTEINS



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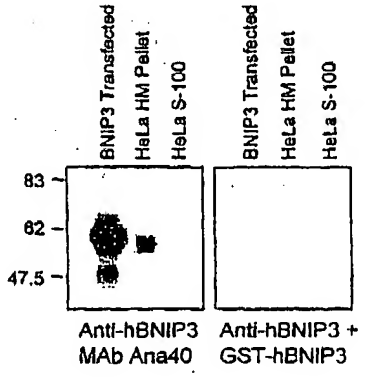

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(57) Abstract: There is provided an isolated and purified BNIP3 protein. There is also provided a method for inducing necrosis in cells by transfecting a cell with a BNIP3 protein. A method for inducing necrosis in cells by creating a transgene overexpressing the gene for the BNIP3 protein is also provided. A transgenic animal and progeny thereof is provided having an expression factor consisting essentially of a BNIP3 protein selected from the group consisting essentially of Seq. ID 1 and 2. Also provided is a vector for use in treating disease including BNIP3 or DN NIP and a promoter. A gene therapy having DNA encoding BNIP3 or DN NIP and a promoter, whereby said gene therapy is used to induce necrosis or inhibit necrosis is also provided.

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THE NIP3 FAMILY OF PROTEINS

BACKGROUND OF THE INVENTION

1. TECHNOLOGICAL FIELD OF THE INVENTION

The present invention is in the field of gene therapy. More specifically, the present invention relates to the field of gene therapy as used to induce necrosis-like cell death in tumorogenic cells.

2. DESCRIPTION OF RELATED ART

Cell death is an important aspect during the embryonic or post-natal development of major organ systems. Apoptosis, or programmed cell demise, also plays a critical role in maintaining homeostasis in many adult tissues. Within vertebrates, bcl-2 is the best understood gene in a cell death pathway and functions as a cell death repressor.

Apoptosis is a term used to refer to the process(es) of programmed cell death and has been described in several cell types (Waring et al. (1991) Med. Res. Rev. 11: 219; Williams G.T. (1991) Cell 65: 1097; Williams G.T. (1992) Trends Cell Biol. 2: 263; Yonisch-Rouach et al. (1991) Nature 352: 345). Apoptosis is likely involved in controlling the amount of and distribution of certain differentiated cell types, such as lymphocytes and other cells of the hematopoietic lineage. The mechanism(s) by which apoptosis is produced in cells is incompletely understood, as are the regulatory pathways by which the induction of apoptosis occurs.

Apoptosis was first described as a morphologic pattern of cell death characterized by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation (Kerr et al., 1992). One hallmark pattern early in the process of cell death is internucleosomal DNA cleavage (Wyllie, 1980). The death-sparing effects of interrupting RNA and protein synthesis and the stereotyped patterns of cell death during development were consistent with a cell autonomous genetic program for cell death (Wyllie et al. (1980) Int. Rev. Cytol. 68: 251; Sulston, J. and Horvitz, H. (1977) Develop. Biol. 56: 110; Abrams et al. (1993) Development

117: 29). The isolation of mutants defective for development cell death in the nematode Caenorhabditis elegans supported this view (Ellis, H. and Horvitz, H. (1986) Cell 44: 817; Hengartner et al. (1992) Nature 365: 494).

The consistency of the morphologic and biochemical patterns defined as apoptosis within different cell types and species, during normal development and as a response to external stimuli are consistent with a common cause of cellular mortality. This thesis is supported by the concept of an endogenous program responsible for cell death and the presence of gene products which are positive and negative regulators of apoptosis. The best studied negative regulator of apoptosis is the bcl-2 proto-oncogene product. It provides the strongest evidence for a shared mammalian pathway of death by its ability to block a wide variety of cell death models.

This pattern of morphologic cell death is characterized by a dramatic plasma membrane blebbing, cell volume contraction, nuclear pyknosis, and internucleosomal DNA degradation following the activation of an endonuclease. Over expression of mitochondrial bcl-2 appears to function as an antidote to this process and has the unique function of blocking programmed cell death independent of promoting proliferation.

The maintenance of homeostasis in normal tissue, in many respects, reflects a simple balanced equation of input (cellular proliferation and renewal) versus output (cell death). This is most easily envisioned for encapsulated organs, such as the prostate, but is also true of the recirculating hematopoietic lineages. The maintenance of remarkably invariant cell numbers reflects tightly regulated death pathways as well as controlled proliferation. See for example S.J. Korsmeyer "bcl-2 Initiates a New Category of Oncogenes: Regulators of Cell Death", Blood Vol. 80 No. 4 pp. 879-886, Aug. 15, 1992.

Programmed cell death represents a cell autonomous suicide pathway that helps restrict cell numbers. The well-defined loss of specific cells is crucial during embryonic development as part of organogenesis. In the mature tissues, genetically programmed demise regulates the volume of cells. A morphologically distinct and temporally regulated cell death entitled apoptosis has been identified by Wyllie A. H.: "Apoptosis: Cell death in tissue regulation". J. Pathol 153: 313, 1987. Cells dying by apoptosis display marked plasma membrane blebbing, volume contraction, nuclear

condensation, and the activation of an endonuclease that cleaves DNA into nucleosomal length fragments.

The genetic regulation of cell death is thought to be a central mechanism of cellular homeostasis and development (Bernardi, P. et al, 1999, Bossy-Wetzel, E. et al, 1998, Boyd, J. M. et al, 1994, Chautan, M. et al, 1999). The Bcl-2 family of genes (Bernardi, P. et al, 1999, Chen, G. et al, 1999), which are related to *ced*-9 of *C. elegans* (Chen, G. et al, 1997), were originally identified as repressors of cell death. It is known that both pro- and anti-apoptotic Bcl-2 homologs exist, however their exact biochemical function has not been determined. Recent studies suggest that Ced-9 and Bcl-2/Bcl-X_L may physically interact with proteins that are required for the execution of apoptosis, Ced-3 and Ced-4 (Chi, S. et al, 1999, Crompton, M. 1999, Datta, S. R. et al, 1997), however these proteins have not been isolated and purified. Ced-3 is a protease which in mammals is represented by a large family of cysteine proteases which cleave after aspartic acid, now called caspases (Chautan, M. et al, 1999, Deas, O. et al, 1998). In mammalian cells overexpression of *bcl*-2 prevents the processing and activation of caspase-3 (CPP32)(Earnshaw, W. C. et al, 1999, Finucane, D. M. et al, 1999).

Bcl-2 family members bear C-terminal transmembrane domains that allow their association with the outer mitochondrial membrane (Goping, I. S. et al, 1998) and this mitochondrial localization is important for the suppressive function of Bcl-2 (Green, D. R. et al, 1998, Griffiths, G. J. et al, 1999). There is growing evidence that mitochondrial function is disturbed early in the apoptotic response and may be important in mediating apoptosis (Gross, A. et al, 1999, Hakem, R. et al, 1998, Harada, H. et al, 1999). This is often seen as the loss of mitochondrial membrane potential (Gross, A. et al, 1999, Hakem, R. et al, 1998) and the release of cytochrome c (Harada, H. et al, 1999), and cytochrome c has been implicated in the activation of caspase (Harada, H. et al, 1999, Horvitz, H. R. 1999, Imazu, T. et al, 1999). Bcl-2 can suppress the release of cytochrome c from mitochondria and prevent caspase activation (Horvitz, H. R. 1999, Imazu, T. et al, 1999).

Additionally, the protein encoded by the bcl-2 proto-oncogene has been reported to be capable of inhibiting apoptosis in many hematopoietic cell systems. The proto-oncogene bcl-2 was isolated and characterized as a result of its frequent translocation adjacent to the immunoglobulin heavy chain enhancer in the t(Green,

D. R. et al, 1998; Harada, H. et al, 1999) chromosome translocation present in more than 80% of human follicular lymphomas (Chen-Levy et al. (1989) Mol. Cell. Biol. 9: 701; Clearly et al. (1986) Cell 47: 19). These neoplasias are characterized by an accumulation of mature resting B cells presumed to result from a block of apoptosis which would normally cause turnover of these cells. Transgenic mice expressing bcl-2 under the control of the Eμ enhancer similarly develop follicular lymphomas which have a high incidence of developing into malignant lymphomas (Hockenbery et al. (1990) Nature 348: 334; McDonnell T.J. and Korsmeyer S.J. (1991) Nature 349: 254; Strasser et al. (1991) Cell 67: 889).

The capacity of bcl-2 to enhance cell survival is related to its ability to inhibit apoptosis initiated by several factors, such as cytokine deprivation, radiation exposure, glucocorticoid treatment, and administration of anti-CD-3 antibody (Nunez et al. (1990) op.cit; Hockenbery et al. (1990) op.cit; Vaux et al. (1988) op.cit; Alnemri et al. (1992) Cancer Res. 52: 491; Sentman et al. (1991) Cell 67: 879; Strasser et al. (1991) op.cit). Upregulation of bcl-2 expression also inhibits apoptosis of EBV-infected B-cell lines (Henderson et al. (1991) Cell 65: 1107). The expression of bcl-2 has also been shown to block apoptosis resulting from expression of the positive cell growth regulatory proto-oncogene, c-myc, in the absence of serum or growth factors (Wagner et al. (1993) Mol. Cell. Biol. 13: 2432). However, the precise mechanism(s) by which bcl-2 is able to inhibit apoptosis is not yet fully defined.

The bcl-2 proto-oncogene is rather unique among cellular genes in its ability to block apoptotic deaths in multiple contexts (Korsmeyer, S. (1992) Blood 80: 879). Overexpression of bcl-2 in transgenic models leads to accumulation of cells due to evasion of normal cell death mechanisms (McDonnell et al. (1989) Cell 57: 79). Induction of apoptosis by diverse stimuli, such as radiation, hyperthermia, growth factor withdrawal, glucocorticoids and multiple classes of chemotherapeutic agents is inhibited by bcl-2 *in vitro* models (Vaux et al. (1988) Nature 335: 440; Tsujimoto, Y. (1989) Oncogene 4: 1331; Nunez et al. (1990) J. Immunol. 144: 3602; Hockenbery et al. (1990) Nature 348: 334; Sentman et al. (1991) Cell 67: 879; Walton et al. (1993) Cancer Res. 53: 1853; Miyashita, T. and Reed, J. (1993) Blood 81: 151). These effects are proportional to the level of bcl-2 expression. Additionally, the endogenous pattern of bcl-2 expression is highly suggestive of a role in the regulation of cell survival *in vivo* (Hockenbery et al. (1991) Proc. Natl. Acad. Sci.

USA 88: 6961; LeBrun et al. (1993) Am. J. Pathol. 142: 743). The bcl-2 protein seems likely to function as an antagonist of a central mechanism operative in cell death.

Additionally, Kerr et al. (Kerr, J. F. R. et al, 1972), on the basis of distinct morphological criteria, identified apoptosis as a programmed and intrinsic cell death pathway, in contrast to necrosis, which was viewed as a passive response to injury. It is now clear that apoptosis is a highly regulated genetic program that is evolutionarily conserved in multicellular organisms and is essential for development and tissue homeostasis (Horvitz, H. R. 1999, 57). The genetic program results in the activation of cysteine aspartyl proteases (caspases) that cleave nuclear and cytoplasmic substrates and disassemble the cell (Earnshaw, W. C. et al, 1999, 54), yielding the characteristic morphological features such as chromatin condensation, DNA fragmentation, plasma membrane blebbing, and the formation of apoptotic bodies (Xue, L.Z., et al., 1999). In contrast to apoptosis, necrosis is considered an unregulated process occurring in response to toxicants and physical injury. This form of cell death is morphologically characterized by extensive mitochondrial swelling, cytoplasmic vacuolation, and early plasma membrane permeability without major nuclear damage (Kerr, J. F. R. et al, 1972, Kitanaka, C. et al, 1999, 55).

Mitochondria appear to play a central role in the induction of cell death. This is thought to occur by at least three possible mechanisms: (i) release of apoptogenic proteins that facilitate caspase activation, (ii) disruption of electron transport, oxidative phosphorylation, and ATP production that can result in an energetic catastrophe, and (iii) alteration of the redox potential, resulting in increased cellular oxidative stress (Green, D. R. et al, 1998). The main biochemical determinant of apoptosis is the activation of caspases, and this is in part regulated by mitochondria. All caspases are synthesized as an inactive polypeptide (zymogen) that must be proteolytically processed to form an active tetramer (Earnshaw, W. C. et al, 1999). Recent work proposes that this processing is initiated through autocatalytic activation. For example, the caspase 8 zymogen is aggregated for autoprocessing by ligand-induced clustering of trimeric death receptors such as CD95/ Fas (Srinivasula, S.M., et al., 1998). Active caspase 8 cleaves the proapoptotic BCL-2 family member BID, which is then able to translocate to mitochondria (Li, H. et al, 1998, Luo, X. et al, 1998). BID, as well as many other apoptotic signals, induces

mitochondria to release cytochrome c, which functions as a cofactor with dATP for Apaf-1 binding and activation of caspase 9 and downstream effector caspases (Li, P. et al, 1997, 51). Another less well studied mitochondrial apoptogenic protein is apoptosis-inducing factor (AIF), a flavoprotein released in response to apoptotic signals that translocates to the nucleus to induce DNA fragmentation and chromatin condensation in a caspase-independent manner (Tsujimoto, Y. 1997).

Apoptotic cell death signals induce other mitochondrial changes, such as opening of the permeability transition (PT) pore, a putative highly regulated ion channel located at the contact sites between the inner and outer mitochondrial membrane (Crompton, M. 1999). The PT pore is a large protein complex, primarily composed of the adenine nucleotide transporter (ANT), cyclophilin D, and voltage-dependent anion channel (VDAC [also called porin]), that can interact with several other proteins (Crompton, M. 1999, Kroemer, G. et al, 1998). When the PT pore is in the open state, it permits the passage of solutes of ;1,500 Da and results in depolarization of mitochondria, which consequently decreases the measured proton electrochemical gradient (Dcm). This, in turn, can lead to the inhibition of respiration, generation of reactive oxygen species (ROS), and loss of ATP production (Bernardi, P. et al, 1999, Crompton, M. 1999). PT pore opening also increases the permeability of certain ions across the mitochondrial membrane, resulting in increased water influx into the matrix and consequent large-amplitude mitochondrial swelling (Gross, A. et al, 1999, Lemasters, J. J. et al, 1998).

The biochemical determinants of necrotic cell death are less well defined, but similar to apoptosis. It has been suggested that the PT pore might play a major role in necrosis. PT pore opening has been described in response to a rise in cytosolic free Ca 21 , anoxia, and reperfusion oxidative stress with overproduction of ROS in cardiac myocytes (Crompton, M. 1999). Although both apoptosis and necrosis are associated with PT pore opening, necrosis is distinguished by an early loss of plasma membrane integrity and ATP, whereas both are maintained and ATP production is required for apoptosis (Leist, M. et al, 1997, Nicotera, P. et al, 1998).

In 1996 Dr. A.H. Greenberg's lab, isolated a protein called BNIP3 and soon thereafter determined that a homodimeric complex of BNIP3 was associated with the energy producing organelle of the cell, the mitochondria, and that BNIP3 had a function in cell death (Chen G et al, 1997). Along with human BNIP3, other family

members NIX and NOX (NINA) were identified, as were homologues from other species namely mouse and *c. elegans* (Chen G et al, 1999; Cizeau J et al, 2000). The functional domains of BNIP3 were characterized (shown in Figure 14) and a series of structural and functional mutants were constructed (shown in Table 1) (Ray R et al, 2000). All of these mutants exhibit a characteristic structural or functional loss. Some, for example BNIP3DTM, can act as dominant negatives, thereby interfering with the natural function of BNIP3, while other construct products have a point mutation that totally eliminates the cell death function of BNIP3. Initially it was believed that BNIP3 was somehow involved in apoptosis; however, subsequent work on the biological mechanism of BNIP3 revealed that this was inaccurate (Vande Velde C et al, 2000).

It would therefore be useful to determine the role BNIP3 plays in cell death. It would also be useful to determine methods of using BNIP3 to treat diseases.

SUMMARY OF THE INVENTION

According to the present invention, there is provided an isolated and purified BNIP3 protein. There is also provided a method for inducing cell death in cells by transfecting a cell with a BNIP3 protein. A method for inducing cell death in cells by creating a transgene overexpressing the gene for the BNIP3 protein is also provided. A transgenic animal and progeny thereof is provided having an expression factor consisting essentially of a BNIP3 protein selected from the group consisting essentially of Seq. ID 1 and 2. Also provided is a vector for use in treating disease including BNIP3 or DN NIP and a promoter. A gene therapy having DNA encoding BNIP3 or DN NIP (or a BNIP3 delta TM) and a promoter, whereby said gene therapy is used to induce cell death or inhibit cell death is also provided.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figures 1A through C are photographs showing BNIP3 expression and integration into mitochondrial membranes;

Figures 2A and B are graphs showing that the broad spectrum caspase inhibitors Ac-zVAD.FMK baculovirus P53 failed to inhibit BNIP3 induced cell deaths;

Figures 3A and B are graphs showing that BNIP3 does not activate caspases;

Figures 4A and B show that BNIP3 does not induce mitochondrial cytochrome c release;

Figures 5A and B are graphs showing BNIP3 induced cell death in the absence of a PAF-1, caspase-9, or caspase-3;

Figures 6A and B are graphs that BNIP3 induces rapid plasma membrane permeability but not PE externalization;

Figures 7A and B are graphs showing BNIP3 induced cell death is characterized by late DNA fragmentation;

Figures 8A through F are photographs showing BNIP3 induces the ultrastructural changes of necrosis;

Figures 9A through E are photos and graphs BNIP3 induced cell death is characterized by mitrochondrial dysfunction;

Figures 10A through E show the inhibition of BNIP3 induced mitrochondrial dysfunction cell death by PT inhibitors;

Figure 11 shows a model of BNIP3 induced cell death;

Figure 12 is a photograph showing the neonatal cardiac myocytes which were incubated in hypoxic(+) or normoxic(-) conditions for twenty-four hours;

Figure 13 is a graph showing BNIP3 induce cell death is inhibited by BNIP3 DN; and

Figure 14 shows the functional domains of hBNIP3.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides a method of producing a purified BNIP3 protein, Seq. ID No:1 through 7, and functional analogs thereof and vectors formed containing therein the BNIP3 protein or variations thereof. Also, provided is a method of inducing or inhibiting necrosis-like cell death in cells by transfecting a cell with a BNIP3 protein or variations thereof. A detailed description of the isolation protocols and its properties are set forth in the Experimental Section.

Biological mechanism studies revealed two important aspects of BNIP3's biological function. First, the studies revealed that alone, high levels of BNIP3 expression and subsequent high levels of BNIP3 protein were not necessarily sufficient for the biological activity of BNIP3. More importantly, the type of association of BNIP3 with the mitochondrial membrane dictated the biological functionality. Cell death would occur only when BNIP3 was tightly associated with the mitochondrial membrane. The second important finding from the biological mechanism studies was that BNIP3 cell death did not involve the caspase cascade; therefore, by definition this death was not apoptosis but necrosis.

By functional analogs, it is meant that an analog will generally be at least 70% homologous over any portion that is functionally relevant. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the BNIP3 protein. The amino acid sequence of an analog may differ from that of the BNIP3 protein when at least one residue is deleted, inserted or substituted. Differences in glycosylation can provide analogs. The molecular weight of the BNIP3 protein can vary between the analog and the present invention due to carbohydrate differences.

Many apoptotic signaling pathways are directed to mitochondria, where they initiate the release of apoptogenic proteins and open the proposed mitochondrial permeability transition (PT) pore that ultimately results in the activation of the caspase proteases responsible for cell disassembly. BNIP3 (formerly NIP3) is a member of the Bcl-2 family that is expressed in mitochondria and induces apoptosis without a functional BH3 domain. It was reported that endogenous BNIP3 is loosely associated with mitochondrial membrane in normal tissue but fully integrates into the mitochondrial outer membrane with the N terminus in the cytoplasm and the C terminus in the membrane during induction of cell death. BNIP3-mediated cell death is independent of Apaf-1, caspase activation, cytochrome *c* release, and nuclear translocation of apoptosis-inducing factor. However, cells transfected with BNIP3 exhibit early plasma membrane permeability, mitochondrial damage, extensive cytoplasmic vacuolation, and mitochondrial autophagy, yielding a morphotype that is typical of necrosis. These changes were accompanied by rapid and profound mitochondrial dysfunction characterized by opening of the mitochondrial PT pore, proton electrochemical gradient ($\bar{D}cm$) suppression, and increased reactive oxygen

species production. The PT pore inhibitors cyclosporin A and bongkrekic acid blocked mitochondrial dysregulation and cell death. *BNIP3* is a gene that mediates a necrosis-like cell death through PT pore opening and mitochondrial dysfunction.

BNIP3 is a member of a unique subfamily of death-inducing mitochondrial proteins that includes NIX (also called BNIP3a and BNIP3L/B5) (Chen, G. et al, 1999, Matsushima, M. et al, 1998, Ohi, N. et al, 2000, 61) and a *Caenorhabditis elegans* ortholog, ceBNIP3 (61; J. Cizeau and A. H. Greenberg, submitted for publication). BNIP3 family members contain a C-terminal transmembrane (TM) domain that is required for mitochondrial localization as well as for its proapoptotic activity (Chen, G. et al, 1999, Chen, G. et al, 1997, 62). Many members of the BCL-2 family require a BCL-2 homology 3 (BH3) domain to induce apoptosis. BNIP3 contains a sequence that resembles a BH3 domain (amino acids 110 to 118) (Zamzami, N., et al., 1995). However, in the context of the BNIP3 protein, it was shown that it is not required for heterodimerization with BCL-2 family members or cell death, both *in vivo* and *in vitro* (Shimizu, S., et al., 1999), indicating that BNIP3 does not trigger apoptosis, like most BH3-containing proteins.

Studies of the biological mechanism of BNIP3 induced cell death has revealed two important aspects of BNIP3's biological function. First, the studies revealed that alone, high levels of BNIP3 expression and subsequent high levels of BNIP3 protein were not necessarily sufficient for the biological activity of BNIP3. More importantly, the type of association of BNIP3 with the mitochondrial membrane dictated the biological functionality. Cell death would occur only when BNIP3 was tightly associated with the mitochondrial membrane. The second important finding from the biological mechanism studies was that BNIP3 cell death did not involve the caspase cascade; therefore, by definition this death was not apoptosis but necrosis. There can now three categories of cell death: 1) apoptosis, 2) necrosis, and now 3) regulated necrosis.

This BNIP3 induced cell death is caspase independent and characterized by early plasma membrane and mitochondrial damage, before the appearance of chromatin condensation or DNA fragmentation. BNIP3 induces rapid opening of the mitochondrial PT pore accompanied by Dcm suppression and increased ROS production. These changes and BNIP3-induced cell death are blocked by the PT pore inhibitors cyclosporin A and bongkrekic acid. BNIP3 activates a novel caspase-

independent necrosis-like cell death pathway, which is mediated through the opening of the PT pore. Accordingly, controlling the expression of the BNIP3 protein enables one to control necrosis-like cell death. The control can either be in the form of inducing cell death or inhibiting cell death.

There are a number of therapies which may be accomplished using the BNIP and DN NIP proteins. For example, the proteins can deliver a necrosis-like cell death signal to cancer cells or other cells that one wishes targeted for elimination. It can be delivered *ex vivo* (bone marrow), by direct intratumoral delivery or via a targeting vector *in vivo*. Delivery by viral vector or possibly raw DNA, or by small peptides (or molecular derivatives) based on the protein structure can also be done.

The anti-apoptotic action of the DN NIP mutant(s), which are generic dominant negative forms of the protein such as BNIP3 delta TM, can be used for tissue (eg. bone marrow) protection during chemotherapy or immunotherapy. Ischemic diseases (eg. myocardial infraction, stroke) have an important apoptotic component that may be blocked by the NIP3 mutant. It can be used in organ or tissue transplantations where implanted tissues can be first treated with the gene to prevent subsequent immune attack. The present invention therefore provides methods of either inducing or inhibiting cell death by controlling the expression of the BNIP3 protein. The method can be used in treating cancer, neurological diseases, and cardiovascular diseases. The cardiovascular diseases to which this method pertains includes, by is not limited to acute and chronic cardiac conditions such as, cardiac hypoxia, cardiac hypoxia-reoxygenation, cardiac ischemia-reperfusion injury, ischemic heart disease, heart failure (including congestive heart failure), heart hypertrophy (all stages), by-pass surgery, coronary angioplasty, vascular defects (atherosclerosis), congenital heart (defects) disease, and cardiac cell muscle regeneration.

More specifically, enhanced BNIP3 death can be triggered by increasing BNIP3 expression and/or by increasing tight association of BNIP3 with the mitochondrial membrane. Enhanced BNIP3 function also proves to be important in the treatment of certain genetic disorders. Since regulated cell death is critical in embryonic development, any disruption of cell death pathways results in genetic defects. Through the characterization of regulated necrosis involving BNIP3, it is now possible that certain genetic dysfunctions can be traced back to a defect in this

regulated necrotic pathway. By compensating for this loss of BNIP3 function, it is possible to overcome the genetic anomaly.

In addition to enhancing BNIP3 function, decreasing its function can also have significant clinical relevance. There are various clinical conditions recognized where necrotic cell death has been identified. Examples of this are cardiac cell death caused by hypoxia during heart attacks or neuronal cell death caused by trauma to the brain. Both of these clinical conditions have been shown to involve an increased expression and tight mitochondrial association of BNIP3. Therefore by preventing BNIP3 action through a decrease in its expression or tight mitochondrial association, it is possible to limit cardiac damage during heart attacks as well as neuronal damage cause by brain trauma.

The above discussion provides a factual basis for the use of BNIP3, DN NIP, and mutants thereof in controlling cell death. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

METHODS:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual Series, Vols. 1-4* Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.)

General methods in immunology:

Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunoassays

In general, ELISAs are the preferred immunoassays employed to assess a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989

Antibody Production

Antibody Production: Antibodies can be either monoclonal, polyclonal or recombinant. Conveniently, the antibodies can be prepared against the immunogen or portion thereof for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof can be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is

monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

For producing recombinant antibody (see generally Huston et al, 1991; Johnson and Bird, 1991; Memaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (CDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, b-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Recombinant Protein Purification

Marshak et al, "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press, 1996.

Transgenic and Knockout Methods

The present invention can provide for transgenic gene and polymorphic gene animal and cellular (cell lines) models as well as for knockout models. These models are constructed using standard methods known in the art and as set forth in United States Patents 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384,5,175,383, 4,736,866 as well as Burke and Olson (1991), Capecchi (1989), Davies et al. (1992), Dickinson et al. (1993), Duff and Lincoln (1995), Huxley et al. (1991), Jakobovits et al. (1993), Lamb et al. (1993), Pearson and Choi (1993), Rothstein (1991), Schedl et al. (1993), Strauss et al. (1993). Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide information.

For gene therapy:

By gene therapy as used herein refers to the transfer of genetic material (e.g DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. Alternatively, the genetic material of interest encodes a suicide gene. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* [Culver, 1998]. These genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression vehicle is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression vehicle can include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art. It should be noted that often the 5'UTR and/or 3'UTR of the gene can be replaced by the 5'UTR and/or 3'UTR of the expression vehicle. Therefore as used herein the expression vehicle can, as needed, not include the 5'UTR and/or 3'UTR of the actual gene to be transferred and only include the specific amino acid coding region.

The expression vehicle can include a promotor for controlling transcription of the heterologous material and can be either a constitutive or inducible promotor to allow selective transcription. Enhancers that can be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any non-translated DNA sequence which works contiguously with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The expression vehicle can also include a selection gene as described herein below.

Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature.

Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be

modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation do not occur.

Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention depends on desired cell type to be targeted and is known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed do not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector depends upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative

diseases. Following injection, the viral vectors circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients or into the spinal fluid. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Delivery of gene products/therapeutics (compound):

The compound of the present invention is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

In the method of the present invention, the compound of the present invention can be administered in various ways. It should be noted that it can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial,

intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants of the compounds are also useful. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses can be single doses or multiple doses over a period of several days, but single doses are preferred.

The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

When administering the compound of the present invention parenterally, it is generally formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and

the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver it orally or intravenously and retain the biological activity are preferred.

In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered can vary for the patient being treated and can vary from about 100 ng/kg of body weight to 100 mg/kg of body weight per day and preferably can be from 10 mg/kg to 10 mg/kg per day.

Example 1

MATERIALS AND METHODS

Cell lines.

MCF-7 and HeLa cells were cultured in a minimal essential medium (MEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (Cansera), 1% MEM sodium pyruvate (Gibco-BRL), 1% HEPES (Gibco-BRL), and 1% L-glutamine (Gibco-BRL). Mouse embryonic fibroblasts (MEFs) deficient in Apaf-1, caspase 9, or caspase 3 were cultured as previously described (Hakem, R. et al, 1998). 293T and 293-Bcl-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% FBS.

Expression plasmids.

T7-tagged pcDNA3-BNIP3, T7-tagged pcDNA3-BNIP3DTM (Chen, G. et al, 1997), and HA-tagged pcDNA3-BNIP3 (Chen, G. et al, 1999) have been described previously. pcDNA3-caspase-9-His6 and pcDNA1-p35 were gifts from Emad Alnemri (Thomas Jefferson University, Philadelphia, Pa.). pcDNA3-Apaf-1 and pFLAG-CMV-5a-tBID were provided by Xiaodong Wang (Howard Hughes Medical Institute, Dallas, Tex.) and Junying Yuan (Harvard Medical School, Boston, Mass.), respectively.

Reagents.

Murine monoclonal anti-T7 antibody was purchased from Novagen (Madison, Wis.). Murine monoclonal anti-cytochrome *c* antibodies for immunoblotting (65981A) and immunofluorescence (67971A) were purchased from Pharmingen. Mouse monoclonal anti-poly(ADP-ribose) polymerase, anti-BCL-XL, and antiactin antibodies were purchased from Alexis Biochemicals (San Diego, Calif.), Transduction Laboratories (Lexington, Ky.), and ICN Biochemicals (Montreal, Canada), respectively. Rabbit polyclonal anti-AIF was a gift from Guido Kroemer (CNRS, Paris, France). Rabbit anti-FLAG polyclonal antibody and mouse anti-HA monoclonal antibody were purchased from Zymed (South San Francisco, Calif.) and Boehringer Mannheim (Indianapolis, Ind.), respectively. Secondary antibodies, goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase, goat anti-mouse IgG-fluorescein isothiocyanate (FITC), and goat anti-rabbit IgG-FITC were all purchased from Sigma Chemical Co. (St. Louis, Mo.). Goat anti-mouse IgG-Cy3 was from Chemicon (Temecula, Calif.).

Assessment of mitochondrial protein targeting and orientation.

MCF-7 and 293T cells (106) were transiently transfected with LipofectAmine reagent (Gibco-BRL) with 8 mg of DNA for 12 hours. Mitochondria were isolated according to Goping et al. (Goping, I. S. et al, 1998) with modifications. Briefly, at

4°C, thigh muscle from the mouse hind limb or transfected cells were isolated or scraped, respectively, and washed twice in 5 ml of HIM (0.2% [wt/vol] bovine serum albumin, 200 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, 1 mM EGTA [pH 7.5]). Cells were resuspended in 2 ml of HIM and homogenized on ice three times for 3 to 10 seconds using a Polytron homogenizer (setting 6.5). Large cellular debris was removed from the homogenate via centrifugation at 430 3 *g* for 10 minutes. The supernatant was diluted in HIM (minus bovine serum albumin), and mitochondria were collected by centrifugation at 5,400 3 *g* for 10 minutes and resuspended in cMRM (250 mM sucrose, 10 mM HEPES-KOH, 1 mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K2 HPO4 [pH 7.5]) to 1 mg of mitochondrial protein per ml and adjusted to 1 mM dithiothreitol just prior to use.

To assess the association of proteins with the mitochondrial membrane, 30 or 100 mg of mitochondria isolated from transfected cells or tissue, respectively, were pelleted and resuspended to 0.25 mg of protein per ml in freshly prepared 0.1 M Na2 CO3 (pH 11.5) and incubated on ice for 30 minutes (Goping, I. S. et al, 1998). Mitochondrial membranes were collected via ultracentrifugation at 100,000 3 *g* for 1 hour at 4°C in a Beckman Optima TLX ultracentrifuge (Beckman Instruments, Fullerton, Calif.). Association of the proteins with the mitochondrial membrane was assessed via Western blot analysis of the pellets and the lyophilized supernatants. To determine protein orientation, 293T cells were transiently transfected with T7-tagged BNIP3 and incubated with 3 mg of trypsin (Sigma) per ml for 10 minutes on ice. Trypsin was inactivated with a 100-fold excess of soybean trypsin inhibitor (Sigma). Trypsin-treated mitochondria were pelleted, subjected to alkali elution, and immunoblotted with mouse monoclonal anti-BNIP3 (Ana40) or anti-T7 (Novagen) antibodies.

b-Galactosidase cell death assay.

Various doses of the peptide caspase inhibitor Ac-zVAD-fmk (Enzyme System Products, Dublin, Calif.) were applied to 1 x 10^5 293T cells cotransfected, using LipofectAmine reagent (Gibco-BRL), with 0.01 mg of the reporter plasmid pcDNA3-bgal plus the indicated expression plasmids to a final amount of 0.75 mg of DNA (see Figure 2), as adjusted with empty vector. Cells were fixed, stained, and evaluated 27 hours posttransfection as described previously (Miura, M. et al., 1993). Similar strategies were used to evaluate the expression of pcDNA1-p35 as a

caspase inhibitor (1.95 mg of DNA total) and to determine the killing efficiency of BNIP3 expressed in MEF cells (1.2 mg of DNA with 0.3 mg of b-galactosidase).

Assessment of caspase activation.

Lysates were collected from 293T cells, transiently transfected via the CaPO4 method (Ray, R., et al., 1999), at the indicated times. Aliquots of these lysates were run under Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) conditions and immunoblotted with mouse monoclonal anti-PARP. Results were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Amersham, U.K.). Transfected 293T cells were also assayed for caspase cleavage via colorigenic cleavage of the peptide Ac-DEVD-pNA (Biomol, Plymouth Meeting, Pa.), according to the conditions outlined by Quignon et al. (Scaffidi, C., et al., 1998), using 100 mM peptide. Where appropriate, samples were preincubated with 500 nM Ac-DEVD-fmk for 30 minutes at room temperature. Data were acquired on a Multiskan MCC/340 (Titertek) plate reader at 405 nm.

Assessment of cytochrome *c* release.

Mitochondria were isolated from CaPO4 –transfected 293T cells using 70 strokes (tight pestle) in a 1-ml Dounce homogenizer (Wheaton) in 300 ml of CFS buffer as previously described (Thomberry, N.A., et al., 1998). Mitochondria were resuspended in H buffer (Thornberry, N.A., et al., 1998). Aliquots of 5 mg of protein were analyzed on Laemmli SDS–15% PAGE gels and immunoblotted with anti-cytochrome *c* monoclonal antibody. Equal loading was ensured by probing the same blot with monoclonal antiactin. Results were visualized with enhanced chemiluminescence. Cytochrome *c* release was also determined via indirect immunofluorescence of transfected MCF-7 and 293T cells. Briefly, cells grown on coverslips were costained with Cy3-conjugated mouse anti-cytochrome *c* monoclonal antibody and an appropriate tag (HA for BNIP3 and FLAG for tBID), which was visualized with FITC-conjugated goat anti-rabbit IgG. Cells were also stained with Hoechst dye to determine apoptotic nuclear morphology. No fewer than 200 cells were scored for each sample. Fluorescence was visualized and captured using a Zeiss axiophot microscope equipped with a cooled charge-coupled device camera.

DNA fragmentation assays and annexin V staining.

DNA fragmentation was detected using the *in situ* cell death detection kit with fluorescein (Boehringer Mannheim) as per the manufacturer's recommendations in the presence or absence of 50 mM Ac-zVAD-fmk or Ac-FA-fmk. Images were captured as described earlier. No fewer than 200 nuclei were scored manually for each sample. Annexin V staining was performed exactly as described by the manufacturer (Boehringer Mannheim), and samples were analyzed via flow cytometry.

Assessment of PT pore opening by confocal imaging.

Aliquots of 293T cells were grown on coverslips, and 9 to 10 hours after BNIP3 or control transfections using the CaPO4 method, the cells were washed with Hanks' balanced salt solution–10 mM HEPES (pH 7.2) (HH buffer) before staining with 1 mM calcein- AM ester (Molecular Probes, Eugene, Oreg.) and 5 mM CoCl2 at room temperature for 15 minutes. The CoCl2 was added to quench the cytoplasmic staining so only the fluorescent mitochondria were imaged (Bernardi, P. et al, 1999). Cells were washed four times and resuspended in HH buffer before imaging on an Olympus IX70 inverted confocal laser microscope using Fluoview 2.0 software (Carson Group Inc., Markham, Ontario, Canada). A bandpass filter of 488 nm was used for capturing the calcein images, while Nomarski optics were used to obtain transmitted light images of the cells. To determine the mitochondrial calcein fluorescence levels, individual cells were identified using Nomarski optics and total mitochondrial fluorescence per cell was measured using Northern Eclipse software, version 5.0 (Empix Inc., Toronto, Ontario, Canada).

Mitochondrial Dcm and ROS production.

Changes in mitochondrial function were determined by incubating 10 6 293T cells, transiently transfected via the CaPO4 method, with either 1 mM JC-1, 40 nM DiOC6 ,or 2mM dihydroethidium (HE) (all from Molecular Probes, Inc.) for 30 minutes at 37°C in Hanks' balanced salt solution (Gibco-BRL). Cells were scored using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, Calif.), and data were analyzed on Cellquest software, version 3.1 (Becton-Dickinson). Controls were performed in the presence or absence of 50 mM mCICCP (Sigma) or an excess of 30% H2 O2 . For inhibition experiments, cyclosporin A (Sigma) or bongkrekic acid (a gift from J. A. Duine, Delft University, Delft, The Netherlands) was added 2 hours prior to transfection. All cells were harvested 8 hours after transfection and stained

with 40 nM DiOC6 ,2mM HE, or 1 mg of propidium iodide (PI) (Sigma) per ml. In all cases, samples were gated to exclude cellular debris.

Electron microscopy.

Transfected 293T cells were fixed with 2% paraformaldehyde– 0.1% gluteraldehyde in 0.1 M sodium cacodylate for 1 to 2 hours atroom temperature. Cells were postfixed with 1% osmium tetroxide for 1.5 hours, washed, and block stained for 1 hour in 3%aqueous uranyl acetate. The samples were then washed again, dehydrated with graded alcohol, and embedded in Epon-Araldyte resin (Maynard Scientific). Ultrathin sections were cut on a Reichert ultramicrotome, counterstained with 0.3% lead citrate, and examined on a Philips EM420 electron microscope.

RESULTS

Mitochondrial membrane expression and integration of NIP3.

Polyclonal and monoclonal antibodies to BNIP3 were developed to examine protein expression in tissues and cell lines. Surprisingly, high levels of protein expression were found only in postnuclear lysates of murine and human skeletal muscle but not in other tissues (e.g., thymus, spleen, lung, kidney, heart, and brain) (Figure 1A) or in many human and murine cell lines examined. Following subcellular fractionation, the skeletal muscle BNIP3 protein was recovered in heavy membrane fractions enriched for mitochondria but not in the S-100 cytosol (Figure 1A, left panel). Subsequent reexamination of several human cell lines that had been negative in cell lysates by Western blotting revealed small amounts of BNIP3 protein in purified mitochondrial fractions using monoclonal anti-human BNIP3 (hBNIP3) Ana40. Again, no protein was detected in S-100 cytosol (Figure 1A, right panel). The significance of these observations was unclear, as skeletal muscle is a terminally differentiated tissue and largely unaffected by programmed cell death, and the cell lines were completely viable. However, it did indicate that the endogenous BNIP3 in skeletal muscle must be inactive. Recent studies by Goping et al. (Goping, I. S. et al, 1998) found that endogenous BAX is only loosely associated with the mitochondrial membrane in normal cell lines, but following death signals, it integrates fully and becomes active. The mitochondrial membrane association of BNIP3 in normal

skeletal muscle and following transient transfection and initiation of apoptosis was examined.

The membrane association of BNIP3 was determined following alkali extraction of mitochondria, which dissociates and solubilizes unintegrated protein. BNIP3 in mouse skeletal muscle was exclusively associated with the mitochondrion-enriched heavy membrane pellet in normal buffers (Figure 1A), but following alkali treatment, the majority of the protein was soluble and detected in the S-100 supernatant. In contrast, endogenous BCL-XL from murine muscle remained tightly associated with the heavy membrane fractions following alkali treatment, as expected of an integral membrane protein (Figure 1B, lower panel). A similar experiment was performed using mitochondria derived from 293T or MCF-7 cells transiently transfected with BNIP3. In contrast to the endogenous protein, transfected BNIP3 remained tightly associated with the heavy membrane fractions following alkali elution (Figure 1B).

Integrated BNIP3 has an Ncyto -Cin orientation in the mitochondrial outer membrane.

The orientation of a protein in any membrane can be a contributing factor to its function and regulation (Nguyen, M. et al, 1993). The trypsin cleavage sites in the BNIP3 TM domain (amino acids 164 to 184) were exploited and the epitope recognized by the monoclonal anti-BNIP3 antibody Ana40 (amino acids 112 to 124) to determine the mitochondrial membrane orientation of transfected BNIP3 bearing a C-terminal T7 tag.

There are three possible orientations for BNIP3 in the mitochondrial membrane that can be detected by this method: (i) mitochondrial inner membrane, (ii) mitochondrial outer membrane with an Ncyto -Cin orientation, or (iii) mitochondrial outer membrane with an Nin -Ccyto orientation. Integration of BNIP3 into the mitochondrial inner membrane would prevent exposure to trypsin and thus result in an undigested 40-kDa BNIP3 homodimer detectable by both Ana40 and anti-T7 antibodies. Alternatively, orientation of BNIP3 such that the C terminus is cytosolic would permit cleavage at R185 and R186, yielding a truncated BNIP3 homodimer of ;38 kDa that would be detected by Ana40 but not by anti-T7 antibody since the C-terminal T7 tag would be lost. Finally, a cytosolic N-terminal orientation would yield truncated fragments detectable by one or both antibodies. Following isolation of mitochondria from BNIP3-transfected cells and trypsin digestion, a prominent 18-kDa

band was recognized by both antibodies and an 8-kDa band was detected by the anti-T7 but not the Ana40 antibody (Figure 1C). The 8-kDa band was detected in the heavy membrane pellet fraction. Thus, the 8-kDa band would contain the extreme C-terminal T7 epitope and is likely a dimer of two 4-kDa monomeric C-terminal fragments representing approximately amino acids 154 to 194. This pattern is consistent with the integration of BNIP3 in the mitochondrial outer membrane in the Ncyto -Cin orientation.

BNIP3-induced cell death is caspase independent and does not induce cytochrome *c* release.

To determine if BNIP3-induced cell death was mediated by caspases, the effectiveness of the broad-spectrum peptide caspase inhibitor Ac-zVAD-fmk and the baculovirus antiapoptotic gene p35 were evaluated in ability of preventing BNIP3-induced cell death following transient transfection of 293T cells. BNIP3-induced cell death was unaffected by the same concentration of inhibitor that effectively suppressed either tBID or caspase 9/Apaf-1 transfectants by greater than 50% (Figure 2A). Furthermore, p35 plasmid was similarly ineffective in abrogating BNIP3 cell death at concentrations of up to 1.5 mg, well above the 0.5 mg of p35 plasmid required to block caspase 9/Apaf-1-induced cell death (Figure 2B).

The caspase substrate Ac-DEVD-pNA was used to detect the activation of caspase 3-like proteases in 293T cells transiently transfected with either BNIP3, tBID, or the inactive mutant BNIP3DTM. Cells were harvested at 1, 12, 18, 24, and 36 hours posttransfection, and lysates were prepared and incubated with the substrate. Lysates from cells transfected with either BNIP3 or BNIP3DTM revealed only marginal increases in proteolytic activity and were not inhibited by the caspase inhibitor of the same specificity as Ac-DEVD-fmk (Figure 3A). In contrast, tBID-transfected cells exhibited a fourfold increase in substrate cleavage, and this was blocked by treatment with Ac-DEVD-fmk (Figure 3A). Lysates from BAX transfectants were similar to those of tBID transfectants (data not shown).

Whole-cell lysates of BNIP3-expressing 293T cells collected at 12, 24, 36, and 48 hours posttransfection were immunoblotted for the caspase substrate PARP and found little evidence of proteolysis (Figure 3B) and no processing of procaspase 3 (Figure 3C). In contrast, efficient processing of PARP from 116 to 86 kDa (Figure 3B) and procaspases 3 (Figure 3C), 7, and 9 were detected in lysates from BAX

transfectants. No processing of caspases 7 and 9 was detected in BNIP3 lysates up to 36 hours.

Since BNIP3 integrates into the mitochondrial outer membrane, it can act to initiate cell death by mitochondrial perturbation and the release of cytochrome c, a cofactor for Apaf-1. Indirect immunofluorescence was initially used to examine cytochrome *c* release from cells expressing BNIP3 following transient transfection. BNIP3- and BNIP3DTM-expressing cells, detected by immunostaining for the C-terminal epitope tag, showed no significant cytochrome *c* release in MCF-7 cells, which are caspase 3 deficient, and only very low levels in 293T cells (Figure 4A). On the other hand, 91% of MCF-7 and 71% of 293T cells released cytochrome *c* 48 hours after transfection with tBID, while the level of cell death induced by tBID and BNIP3 was equivalent (Figure 4A). In tBID-transfected cells, cytochrome *c* was released prior to apoptosis, as determined by Hoechst dye staining.

Cytochrome *c* release was reexamined by Western blotting heavy membrane (HM) and S-100 subcellular fractions of 293T cells at 18, 24, and 36 hours posttransfection. A significant increase in cytochrome *c* was seen in the S-100 fractions of tBID but not BNIP3 transfectants at 18 and 24 hours (Figure 4B). Loss of cell viability of tBID and BNIP3 transfectants was equivalent, as determined by trypan blue dye exclusion. The decrease in cytochrome *c* levels in S-100 of tBID-expressing cells at 36 hours was concomitant with extensive cell death. S-100 cytochrome *c* levels in BNIP3-transfected cells were similar to that of the inactive BNIP3DTM and control cells despite a fivefold difference in viability (Figure 4B). A time course revealed that chromatin condensation following BNIP3 transfection preceded the release of cytochrome c, indicating that it could not be responsible for the nuclear changes.

BNIP3 induces cell death in fibroblasts deficient in Apaf-1, caspase 9, or caspase 3.

Since the above experiments suggested that BNIP3 induced cell death without cytochrome *c* release or caspase activation, the function of BNIP3 in cells lacking Apaf-1 or Apaf-1-activated caspases 9 and 3 was examined. Using the b-galactosidase cell death assay, wild-type, Apaf-1 2/2, caspase 9 2/2, and caspase 3 2/2 MEFs were transiently transfected with either BNIP3 or BNIP3DTM. BNIP3 was able to induce cell death (;50%) in the wild-type and all mutant MEF lines tested

(Figure 5A). In contrast, the mutant cells exhibited profound resistance to adriamycin-induced cell death (Figure 5B), confirming an earlier report (Hakem, R. et al, 1998). Immunoblot analysis of whole-cell lysates showed equal expression of BNIP3 and BNIP3DTM in all of the MEF cell lines.

Rapid loss of plasma membrane permeability in BNIP3- transfected cells.

Cells undergoing apoptosis externalize phosphatidylserine (PS) while maintaining an intact plasma membrane (McConkey, D. J. 1998). A time course following BNIP3 transfection identified increased plasma membrane permeability as early as 2 hours posttransfection and did not increase further over the following 12 hours as determined by the failure of cells to exclude PI (Figure 6A). Cells gated to determine annexin binding as a measure of phosphatidylserine externalization in PI 2 populations at 12 hours revealed no increase in annexin staining of BNIP3-transfected in cells that excluded PI, in contrast to cells transfected with tBID, BAX, or caspase 9/Apaf-1 (Figure 6B). BNIP3-expressing cells analyzed at 18 and 24 hours similarly did not show any increase in annexin staining in PI 2 cells. Thus, BNIP3 induces early permeability of the plasma membrane but not PS externalization.

BNIP3 induces late DNA fragmentation that is independent of AIF translocation.

DNA fragmentation and chromatin condensation are hallmarks of caspase-dependent apoptotic cell death and have been consistently seen in BNIP3-transfected cells (Chen, G. et al, 1999, Chen, G. et al, 1997). Since it was demonstrated that plasma membrane was damaged early following BNIP3 expression, the relative rate at which DNA fragmentation occurred was examined using the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay. BNIP3 transfectants showed increasing levels of TUNEL-positive cells over time, but no activity was detected until 18 to 24 hours and maximal levels were not reached until 36 hours, much slower than tBID-induced DNA damage (Figure 7A). This contrasts with the initiation of plasma membrane damage by BNIP3 at 8 hours and its completion by 18 hours. In addition, it was observed that only two or three TUNEL-positive foci in BNIP3-expressing cells, while tBID-transfected cells exhibited much more extensive nuclear fragmentation, with six to ten TUNEL-positive foci per cell (Figure 7B). DNA fragmentation could only be

partially inhibited with 50 mM Ac-zVAD-fmk in BNIP3 transfectants but was nearly completely inhibited in tBID-expressing cells (Figure 7C). No effect was observed in parallel populations treated with 50 mM Ac-FA-fmk. The DNA fragmentation observed by TUNEL staining on agarose gels stained with ethidium bromide was confirmed. An oligonucleosomal ladder was easily detected in tBID transfectants at 18, 24, and 36 hours, while little DNA degradation and ladder formation was observed in BNIP3 transfectants even at 36 hours.

Since wild-type BNIP3-induced chromatin condensation and DNA fragmentation were not completely blocked by treatment with Ac-zVAD-fmk, AIF can also mediate the BNIP3 effects. AIF is a mitochondrial flavoprotein which, in response to an apoptotic stimulus, translocates to the nucleus to induce chromatin condensation and high-molecular weight DNA fragmentation (Tsujimoto, Y. 1997). Immunofluorescence analysis and immunoblotting of heavy membrane fractions of BNIP3-transfected 293T cells at 18, 24, and 36 hours posttransfection found no AIF nuclear translocation despite increases in the proportion of cells with condensed chromatin by Hoechst staining (C. Vande Velde, J. Cizeau, E. Daugas, G. Kroemer, and A. H. Greenberg, unpublished data).

BNIP3-expressing cells have ultrastructural features of necrosis.

To determine the fine ultrastructural features of cells following BNIP3 expression, transmission electron microscopy of 293T cells 24 hours posttransfection was performed. These experiments revealed a nuclear phenotype of lightly dispersed foci of chromatin condensation and heterochromatin (Figure 8B) rather than the globular condensation typical of apoptosis. During a detailed examination of cellular organelles, many rounded mitochondria were detected in which the internal cisternae had been destroyed, while the inner and outer membranes of the mitochondria appeared to be intact in most cells (Figure 8C). The mitochondria did not appear to be undergoing gross swelling. Surprisingly, BNIP3 transfectants were characterized by extensive cytoplasmic vacuolation and dense bodies. High-power examination of these structures revealed a heterogeneous mixture of electronlucent and electrondense regions, many of which appear to be vacuoles and autophagosomes (Figure 8D and E), and some of the autophagic vacuoles contained whorls of membranous material (Figure 8F) that have been observed during autophagic cell death (Yasuda, M., et al., 1998).

BNIP3 induces mitochondrial PT pore opening, loss of Dcm, and increased ROS production.

Since the studies to this point had established that BNIP3 was a mitochondrial outer membrane protein and electron micrographs of BNIP3-transfected cells featured disturbances in mitochondrial structure, it was established that BNIP3 can directly induce mitochondrial dysfunction. Opening of the mitochondrial PT pore often accompanies both apoptotic and necrotic cell death, with the consequent loss of transmembrane potential (Dcm) and respiratory inhibition with ROS production. The status of the PT pore can be determined with the membrane-permeating fluorescent probe calcein-AM, which freely enters mitochondria but cannot exit except through an open PT pore following processing by cellular esterases. Using CoCl2 quenching of cytosolic fluorescence as described by Bernardi et al. (Bernardi, P. et al, 1999), the release of calcein from mitochondria was analyzed by confocal laser microscopy and quantitative image analysis. Following BNIP3 transfection, 293T cells lose mitochondrial calcein staining as early as 8 hours posttransfection (Figure 9A and B), indicating rapid opening of the PT pore.

To determine if BNIP3-expressing cells also decrease their transmembrane potential and produce ROS, cell-permeating lipophilic dyes JC-1 and HE were used and the dyes assessed the staining by flow cytometry using gates established from normal untransfected 293T cells. At 24 hours posttransfection, cells were collected, stained, and analyzed. BNIP3 was almost as efficient as tBID at suppressing Dcm, increasing ROS generation, and inducing cell death (Figure 9C to E). These changes were identified as early as 2 hours posttransfection and did not increase further during 12 hours of analysis, indicating that the mitochondrial dysfunction was maximal and occurred as early as plasma membrane permeability and cell death (Figure 6A).

Inhibition of PT pore opening prevents mitochondrial dysfunction and cell death.

To confirm that the loss of Dcm, increase in ROS production, and the ensuing cell death were the result of opening of the PT pore, the effect of PT pore inhibitors on BNIP3-induced cell death and mitochondrial deregulation was examined using the potentiometric fluorescent probe DiOC6 in combination with HE. BNIP3-expressing cells showed ;55% DiOC6 -low and HE-high cells as detected in the

upper left quadrant of Figure 10D, consistent with previous experiments using JC-1, while BNIP3DTM-transfected cells were not affected compared to untreated controls. As noted earlier, opening of the PT pore can be inhibited by cyclosporin A, which interacts with cyclophilin D, or bongkrekic acid, which binds to the ANT. Treatment of BNIP3 transfectants with either cyclosporin A or bongkrekic acid revealed a dose-dependent reversal in Dcm suppression, ROS generation, and cell death (PI staining) (Figure 10A to D). Maximum suppression was about 50% of that in control cells. Cells were treated with the drugs for 2 hours and washed prior to transfection, a procedure that did not affect BNIP3 expression in the 293T cells (Figure 10E). Addition of either drug during the transfection suppressed BNIP3 expression. The drugs did not affect mitochondrial function and cell death when added after the transfection. BNIP3 physically interacts with Bcl-2 (Boyd, J. M. et al, 1994, Chen, G. et al, 1997), and Bcl-2 and Bcl-XL overexpression can partly suppress BNIP3-induced apoptosis, although this is overcome at high BNIP3 expression levels (Chen, G. et al, 1999, Chen, G. et al, 1997). The effect of Bcl-2 on BNIP3- induced cell death as measured by PI staining was examined and a reduction in plasma membrane damage in Bcl-2-expressing cells was found (Figure 10F).

Referringn specifically to the figures, Figure 1 shows BNIP3 expression and integration into the mitochondrial membrane. Figure 1A, in the feft panel shows mitochondrion-enriched heavy membrane (HM) and S-100 cytosol (S-100) subcellular fractions of mouse tissues were isolated and alkali extracted as described in Materials and Methods, then Westem blotted with polyclonal anti-BNIP3 antibody. BNIP3 lanes are lysates of 293T cells transfected with BNIP3. Right panel: HeLa cells were fractionated as described above, and fractions were Westem blotted with monoclonal anti-BNIP3 antibody Ana40. Nonspecific staining was evaluated by adding GST-hBNIP3 to a parallel incubation mixture. Figure 1B shows that subcellular fractions of hBNIP3-T7-transfected 293T (top) and MCF-7 (middle) cells were alkali extracted and blotted with mouse monoclonal anti-BNIP3 Ana40 antibody. Mouse skeletal muscle tissue prepared in the same manner was blotted for BCL-XL (bottom). Figure 1C shows mitochondrial heavy membrane fractions from hBNIP3-T7-transfected 293T cells were trypsin digested and/or alkali extracted, as described in Materials and Methods, and blotted with either Ana40 mouse monoclonal anti-hBNIP3 or anti-T7 antibodies. Arrows indicate specific antibody-

reactive bands at 40, 18, and 8 kDa. P, heavy membrane pellet; S, S-100 supernatant.

Figure 2 shows that the broad-spectrum caspase inhibitors Ac-zVAD-fmk and baculovirus p35 fail to inhibit BNIP3-induced cell death. Figure 2A shows that 293T cells were transiently cotransfected with the reporter plasmid pcDNA3-bgal and either BNIP3-T7 (F) or inactive mutant BNIP3DTM-T7. Cells transfected with tBID-FLAG (n) or caspase 9-His6 plus Apaf-1 served as positive controls. All groups were treated with increasing concentrations of Ac-zVAD-fmk. Figure 2B shows that in a parallel experiment, 293T cells were transfected as above with increasing concentrations of pcDNA1-p35. At 27 hours posttransfection, cells were fixed, stained, and evaluated for dead cells as described in Materials and Methods. The data represent one of three independent experiments with similar results.

Additionally, Figure 3 shows that BNIP3 does not activate caspases. Figure 3A shows that BNIP3 expression does not activate a DEVDase. Lysates from 293T cells transfected with BNIP3-T7, BNIP3DTM-T7, or tBID-FLAG were harvested at 1, 12, 18, 24, and 36 hours and then incubated with the substrate DEVD-pNA in the presence (solid bars) or absence (shaded bars) of 500 nM Ac-DEVD-fmk. Fold activation was determined as the ratio of transfected cells to untransfected controls. Results are expressed as the mean 6 standard error (SE) from at least three independent experiments. Figure 3B shows that BNIP3 expression fails to activate PARP cleavage. Lysates from BNIP3-T7-, BNIP3DTM-T7-, or BAX-transfected 293T cells were harvested at 12, 24, 36, and 48 hours posttransfection and immunoblotted with mouse monoclonal anti-PARP antibody. Arrows indicate the unprocessed p116 and processed p85 bands. Figure 3C shows that BNIP3 expression fails to activate procaspase 3 processing. Lysates from BNIP3-T7-, BNIP3DTM-T7-, or BAX-transfected 293T cells were harvested 24 hours posttransfection and immunoblotted with mouse monoclonal anti-procaspase 3 antibody. The arrow indicates the unprocessed p32 band. Lane C is the untreated control.

Figure 4 shows that BNIP3 does not induce mitochondrial cytochrome *c* release. More specifically, Figure 4A shows 293T cells transiently transfected with BNIP3-T7, BNIP3DTM-T7, or tBID-FLAG were stained with monoclonal anti-cytochrome *c* antibody and Cy3-labeled anti-mouse IgG antibody then evaluated by fluorescent microscopy. Time course of cytochrome *c* release and apoptosis

following BNIP3-T7 (“), BNIP3DTM-T7 (n), or tBID-FLAG (F) transfection of 293T (left panels) and MCF-7 (right panels) cells is shown. Cytochrome *c* release was scored as the loss of cytoplasmic granular staining. Apoptotic cells were scored based on chromatin condensation following Hoechst staining. The data from three independent experiments are shown as the mean 6 SE for each time point. Figure 4B shows Western blot analysis of the time course of release of cytochrome *c* from mitochondria into S-100 cytosol. Aliquots of 5 mg of heavy membrane (HM) and S-100 fractions from 293T cells transiently transfected with BNIP3, BNIP3DTM, or tBID were harvested at 18, 24, and 36 hours posttransfection and Western blotted with mouse anti-cytochrome *c* antibody (p16). The same membrane was blotted with mouse antiactin antibody (p43) to demonstrate equal loading. Control, untransfected cells.

Figure 5 shows BNIP3-induced cell death in the absence of Apaf-1, caspase 9, or caspase 3. Figure 5A shows wild-type, Apaf-1 2/2 , caspase 9 2/2 , and caspase 3 2/2 MEFs were transiently cotransfected with pcDNA3-bgal vector alone, BNIP3-T7, or BNIP3DTM-T7 and then scored for dead cells as described in Materials and Methods. Results are expressed as the mean 6 SE from three independent experiments. Figure 5B shows that the same cell aliquots of wild-type (WT), Apaf-1 2/2 , caspase 9 2/2 (Casp 9 2/2), and caspase 3 2/2 (Casp 3 2/2) MEFs used for the experiments in panel A were transfected with pcDNA3-bgal and treated with medium or with 3 mg of adriamycin per ml for 24 hours, and dead cells were enumerated in three experiments. N, N-Dimethyl formamide (DMF) was used to dilute the adriamycin.

Figure 6 shows that BNIP3 induces rapid plasma membrane permeability but not PS externalization. Figure 6A shows untransfected and BNIP3-T7-transfected 293T cells which were harvested at 2, 4, 8, and 12 hours posttransfection and stained with PI. PI 1 cells are expressed as the mean 6 SE of three or four experiments for each time point. Figure 6B shows untransfected 293T cells and 293T cells (Figure 6C) transfected with BNIP3-T7 (BNIP3), BNIP3DTM-T7 (DTM), tBID-FLAG (tBID), BAX, or caspase 9/Apaf-1 (C9/A) were harvested at 12 hours posttransfection and stained for annexin V and PI. Cells that were gated as PS 1 PI 2 are expressed as the mean 6 SE of three independent experiments.

Figure 7 shows BNIP3-induced cell death is characterized by late DNA fragmentation. Figure 7A shows the quantification of TUNEL-positive 293T cells transiently transfected with BNIP3-T7, BNIP3DTM-T7, or tBID-FLAG and stained at 18, 24, and 36 hours. Values for BNIP3- and tBID-transfected cells were significantly higher than those for controls at all time points (*P* , 0.01). Figure 7B shows an illustration of transfected cells as in panel A harvested 24 hours posttransfection and stained with the TUNEL reagent (right) or Hoechst dye (left). Figure 7C shows cells which were transfected as in panel A in the absence (open bars) or presence of 50 mM Ac-FA-fmk (solid bars) or 50 mM Ac-zVAD-fmk (hatched bars). Cells were TUNEL stained 24 hours posttransfection, and the percent positive was scored by fluorescent microscopy.

Figure 8 shows that BNIP3 induces ultrastructural changes of necrosis. Normal 293T cells (Figure 8A) and BNIP3-expressing 293T cells (Figure 8B to F) were examined 24 hours posttransfection by transmission electron microscopy. Nuclei of BNIP3-expressing cells exhibited dispersed foci of chromatin condensation and heterochromatin (Figure 8B) compared to control cells (Figure 8A). High-power magnifications of BNIP3 transfectants showed rounded mitochondria with disrupted internal structures (arrows) (Figure 8C), extensive cytoplasmic vacuolation (Figure 8D), autophagosomes (arrows) (Figure 8E), and autophagic vacuoles containing membranous whorls (Figure 8F). (Figures 8A and B), bar, 1 mm; (Figures 8C to F) bar, 0.5 mm.

Figure 9 shows that BNIP3-induced cell death is characterized by mitochondrial dysfunction. Figure 9A shows the untransfected (control), BNIP3-T7 (BNIP3)- and BNIP3DTM-T7 (BNIP3DTM)-transfected 293T cells were harvested 24 hours after transfection and incubated with calcein-AM in the presence of CoCl2 to quench cytoplasmic fluorescence. Cells were visualized by confocal laser microscopy (left) and Nomarski optics (right). Figure 9B shows the quantitation of calcein fluorescence of cells transfected as described for panel A. The percentages of cells measured as low (CALCEIN LO), intermediate (CALCEIN MED), or high (CALCEIN HI) total fluorescence units per cell are shown. The experiment was repeated with similar results. By chi analysis, *P* ,0.001 for the comparison of control versus BNIP3 and BNIP3DTM versus BNIP3. Figure 9C shows the untransfected (control) and BNIP3-T7 (BNIP3)-, BNIP3DTM-T7 (DTM)-, or tBID-FLAG (tBID)-

transfected 293T cells were harvested at 24 hours, stained with JC-1, and analyzed by flow cytometry as a measure of Dcm. JC-1 LO cells were defined as cells that were gated within the same range as those treated with 50 mMClCCP (;99%). BNIP3- and tBID- but not BNIP3DTM-transfected cells were significantly suppressed compared to controls *(P* ,0.01). Figure 9D shows cells treated as in panel C were stained with HE to measure ROS production. HE HI cells were defined as cells that were gated within the same range as those treated with 30% H2 O2 for 15 minutes (;98%). Levels in BNIP3- and tBID-expressing cells were significantly increased compared to untreated controls or BNIP3DTM *(P* , 0.03; the Student *t* test). Figure 9E shows samples from the control and each of the transfections in panel C were trypan blue stained as a measure of cell death. BNIP3- and tBID-transfected cells were significantly increased compared to untreated controls or BNIP3DTM *(P* , 0.01; the Student *t* test).

Figure 10 shows the inhibition of BNIP3-induced mitochondrial dysfunction and cell death by PT pore inhibitors and Bcl-2. Untransfected (control) and BNIP3-T7-transfected 293T cells harvested 8 hours posttransfection were treated with increasing doses of cyclosporin A or 100 mM bongkrekic acid (BA) and stained with DiOC6 (Figure 9A), HE (Figure 9B), or PI (Figure 9C) as described above. BNIP3DTM-T7-transfected 293T cells were used as a negative transfection control. Results are expressed as the mean 6 SE for at least three independent experiments. Figure 9D shows the flow cytometric histograms of HE and DiOC6 staining of BNIP3-transfected cells treated with 50 mM cyclosporin A (CsA) or 100 mM bongkrekic acid (BA). Figure 9E shows the Western blot of BNIP3-transfected cells treated as described above using anti-T7 epitope antibody. Antiactin antibody was used as a loading control. Suppression of DiOC6 levels in BNIP3 cells was significantly inhibited compared to BNIP3 cells at 25 mM *(P* , 0.05) and 50 mM *(P* , 0.02) cyclosporin A and 100 mM bongkrekic acid *(P* , 0.02). Increase in HE fluorescence was inhibited at 25 mM *(P* , 0.03) and 50 mM *(P* , 0.02) cyclosporin A and 100 mM bongkrekic acid *(P* , 0.02). Cell death was significantly suppressed at 50 mM cyclosporin A *(P* , 0.02), and 100 mM bongkrekic acid *(P* , 0.02). (F) BNIP3-induced cell death (solid bars) in 293T cells and 293 cells overexpressing BCL-2 (BCL-2) compared to the inactive BNIP3DTM mutant (open bars). Eight hours following BNIP3 transfection, cells were stained with PI and evaluated by flow cytometry. The

percent dead cells were calculated as the proportion of cells that were PI positive. Equivalent transfection efficiency was obtained in both cell lines, as detected by immunostaining.

Finally, Figure 11 shows a model of BNIP3-induced cell death. Overexpression permits integration of BNIP3 into the outer mitochondrial membrane in an Ncyto -Cin orientation through its TM domain. BNIP3 then initiates permeability transition pore opening and Dcm suppression with increased ROS production in an undefined sequence, leading to cell death. Late DNA fragmentation and chromatin condensation are also induced as a consequence of BNIP3 integration via an unidentified pathway.

DISCUSSION

In the present study, it was determined that BNIP3 is capable of activating a novel form of cell death resembling necrosis as a consequence of mitochondrial PT pore opening. This mechanism is independent of caspases and the Apaf-1/ cytochrome *c* mitochondrial pathway and occurs before the appearance of nuclear damage.

The mitochondrial membrane integration of many proapoptotic BCL-2 family members induces mitochondrial dysfunction, which plays an important role in the cell death pathway. One of the key events in apoptosis is the release of cytochrome c, which functions with dATP as a cofactor for Apaf-1 activation of the caspase cascade (Green, D. R. et al, 1998). There are currently three proposed models to explain the mechanism of cytochrome *c* release: (i) PT pore-induced mitochondrial swelling and subsequent outer membrane rupture (Wyllie, A.H., et al., 1980); (ii) cytochrome *c* exit from the mitochondria through the PT pore (Susin, S.A., et al., 1999a); and (iii) an undefined cytochrome c-specific channel in the mitochondrial outer membrane (Kluck, R. M. et al., 1999). In one model, the PT pore is hypothesized to serve as a conduit for cytochrome *c* release into the cytoplasm. This is supported by experiments that show a direct interaction between BAX and components of the PT pore, including ANT (Marzo, I. et al, Science 1998) and VDAC/porin (Narita, M. et al, 1998), and evidence that BAX can open the pore sufficiently to allow cytochrome *c* release (Susin, S.A., et al., 1999a). In contrast to BAX, BNIP3 does not induce cytochrome *c* release despite evidence of rapid PT

pore opening. Therefore, a model in which opening of the PT pore is sufficient to release cytochrome *c* is not supported by the data. BAX must have other effects on mitochondrial membrane proteins to account for the difference with BNIP3. Although BNIP3 kills without cytochrome *c* release, it has been observed that the BNIP3 homolog NIX/BNIP3L/ BNIP3a/B5 recombinant protein induces cytochrome *c* release from isolated mitochondria (Imazu, T. et al, 1999). The reason for this difference from BNIP3 is not known, but there are clear structural differences between the proteins that can account for this effect.

Another mechanism for cytochrome *c* release occurs as a result of nonselective PT pore-induced mitochondrial matrix swelling and outer membrane rupture (Wyllie, A.H., et al., 1980). Although electron micrographs of BNIP3-transfected 293T cells show mitochondrial rounding and destruction of cristae, the large-amplitude swelling seen during growth factor withdrawal in interleukin-3-dependent FL5.12 cells or Fas-treated Jurkat cells were not observed (Wyllie, A.H., et al., 1980). It is therefor established that cytochrome *c* release and PT pore opening can be completely separated during BNIP3-induced cell death and thus are independent events in the cell death program.

The absence of mitochondrial cytochrome *c* release does not exclude the activation of a caspase-dependent apoptotic pathway. For example, two different death pathways have been described in Fas-induced apoptosis, one of which leads to direct activation of caspase 3 through receptor-activated caspase 8 and does not require cytochrome c, and a second that requires mitochondrial release of cytochrome *c* to activate caspase 3 and apoptosis (Susin, S.A., et al., 1999). BNIP3, on the other hand, requires neither Apaf-1/cytochrome *c* nor the downstream caspases, as BNIP3-induced cell death was unaffected by broad-spectrum caspase inhibitors and was fully functional in MEF cell lines deficient in Apaf-1, caspase 9, or caspase 3. Thus, BNIP3-induced cell death is primarily caspase independent. Induction of caspase-independent cell death has been increasingly observed, and examples include the adenoviral protein E4ORF4 (Lavoie, J. et al., 1998) and cellular proteins PML (Scaffidi, C., et al., 1998), anti-CD2 (Deas, O. et al, 1998), oncogenic Ras (Chi, S. et al, 1999), and FADD (Kawahara, A. et al, 1998). Furthermore, BAX and BAK are able to induce cell death, as opposed to the nuclear changes of apoptosis, in the presence of the general caspase inhibitor Ac-zVAD-fmk

(McCarthy, N. J. et al, 1997, 59). Although cell death can be caspase independent, DNA fragmentation and chromatin condensation following most apoptotic signals require downstream caspases (Earnshaw, W. C. et al, 1999). Nuclei in BNIP3 transfectants exhibit DNA fragmentation and focal chromatin condensation, although these nuclear changes are preceded by loss of plasma membrane integrity, and thus the cells are likely already committed to die. Nevertheless, it is unclear how the nuclear changes are mediated, as there is only minimal DEVDase activation, even at the late time points. Furthermore, DNA fragmentation is only partially inhibited by Ac-zVAD-fmk Immunofluorescence and immunoblotting of subcellular fractions exclude the participation of AIF, a caspase-independent mediator (Tsujimoto, Y. 1997), as it was not translocated from the mitochondria to the nucleus in BNIP3-transfected cells. Ultrastructural analysis of BNIP3-transfected cells revealed that the nuclei have a peculiar mottled appearance, with dispersed foci of chromatin condensation rather than the global large-scale condensation normally observed in caspase-dependent apoptosis.

BNIP3 transfectants exhibit a rapid loss in plasma membrane integrity, and this precedes the appearance of DNA fragmentation detected by TUNEL. In contrast, cells expressing tBID, BAX, and caspase 9/Apaf-1 showed both the expected apoptotic phenotype of an intact plasma membrane and PS externalization (PS 1 PI 2) as well as some cells with rapid plasma membrane disruption. This observation suggests that the primary cause of BNIP3-induced cell death is the loss of membrane integrity, which would be more typical of a necrotic type of cell death. Electron micrograph analysis of BNIP3- transfected cells supports this interpretation. The morphological changes show extensive cytoplasmic vacuolation and mitochondrial deformation with minimal nuclear damage. Similar vacuole formation has been observed in caspase-independent forms of cell death, including anti-CD2-treated cells (Deas, O. et al, 1998), neuronal cells subjected to nerve growth factor withdrawal (Yasuda, M., et al., 1998), and Ac-zVAD-fmk-treated BAX and BAK transfectants (McCarthy, N. J. et al, 1997, 59). BNIP3-expressing cells contain a heterogeneous population of electron-dense and electronlucent vacuoles, some of which appear to be autophagic and are very similar to the structures recently observed in sympathetic neurons after nerve growth factor withdrawal (Yasuda, M., et al., 1998). In this study, autophagic degeneration and vacuole formation were

blocked by treatment with an autophagy inhibitor, 3-methyladenine, but not the caspase inhibitor Ac-zVAD-fmk, and can be similar to BNIP3-induced cell death. BNIP3-mediated cell death also resembles the caspase- and Apaf-1-independent cell death in the interdigital spaces of mouse limb buds, including mottled nuclei and cytoplasmic vacuolation (Chautan, M. et al, 1999). A morphologically similar form of caspase-independent cell death has been reported in the slime mold *Dictyostelium discoideum*, which was also inhibitable by cyclosporin A (Quignon, F., et al., 1998).

Opening of the PT pore, loss of Dcm, and increased ROS production are important contributors to cellular destruction (63) and are early events in both apoptosis and necrosis (Crompton, M. 1999, Kroemer, G. et al, 1998). PT pore opening has also been described in several models of apoptotic cell death as an amplification step that is secondary to initial caspase activation (Bossy-Wetzel, E. et al, 1998, Finucane, D. M. et al, 1999, Marzo, I. et al, 1998). As noted earlier, necrotic cell death is similarly characterized by rapid PT pore opening that can be inhibited by cyclosporin A, which also effectively blocks cell death (Kroemer, G. et al, 1998). BNIP3-induces PT pore opening with Dcm suppression and ROS production occurs concurrently with plasma membrane permeabilization and is blocked by the PT pore-specific inhibitors cyclosporin A and bongkrekic acid. Thus, PT pore opening is a pivotal event for BNIP3-induced cell death. This is summarized in Figure 11. In earlier studies, there remains a controversy as to the sequence of mitochondrial events in cell death. Zamzami et al. (63) demonstrated that ROS were generated only after dissipation of Dcm following dexamethasone treatment of splenic T cells. The loss of mitochondrial membrane potential and ROS production can be both an inducer and a consequence of PT pore opening depending on the death signal (Crompton, M. 1999, Kroemer, G. et al, 1997, 65). Considering the mechanism by which BNIP3 affects the PT pore, it interacts either directly or indirectly with components of the pore, resulting in its opening. Alternatively, BNIP3 targets another protein that suppresses transmembrane potential and induces ROS production, which secondarily opens the PT pore. Thus, although PT pore opening is a key mechanism that mediates BNIP3-induced cell death, the specific mitochondrial proteins that are targeted remain to be identified. Based on the observed function of BNIP3 as a mediator of cell death resembling necrosis when overexpressed, it is

reasonable to postulate that some forms of necrotic cell death can be mediated by endogenous BNIP3.

Recently, increased endogenous BNIP3 mRNA and protein expression has been observed in HeLa cells grown in hypoxic conditions (K. Guo, G. Searfoss, C. Franks, M. Pagnoni, D. Krolikowsky, K. T. Yu, M. Jaye, K. Clark, and Y. Ivashchenko, Proceedings of the AACR Special Conference on Programmed Cell Death Regulation, abstract A-56, 2000). Hypoxia is a well-known inducer of necrotic cell death (Crompton, M. 1999). Thus, it is interesting to speculate that BNIP3 plays a role in mediating death associated with hypoxic stress and possibly other forms of necrotic cell death. Endogenous BNIP3 protein is abundant in murine and human skeletal muscle and is not detectable in lysates of all other nonskeletal muscle-bearing tissues and over 15 cell lines, including myoblasts and differentiated myocytes (D. Dubik and A. H. Greenberg, unpublished data). However, it was found that some (e.g., HeLa, 293T, and K562) but not all (e.g., MCF-7) cell lines have small amounts of BNIP3 protein detectable in enriched mitochondrial fractions. Endogenous muscle BNIP3 is alkali extractable and thus loosely associated and not integrated into the mitochondrial membrane, similar to the observations for endogenous BAX intracellular localization by Goping et al. (Goping, I. S. et al, 1998). When overexpressed, BNIP3 (and BAX) integrates into the mitochondrial membrane through the C-terminal transmembrane domain (amino acids 164 to 184) with the orientation of the protein in an Ncyto -Cin direction. A 17- to 18-kDa portion of the C terminus is detected after trypsin digestion of mitochondria. This would be consistent with a dimer of two trypsin-resistant fragments of 8.5 to 9.0 kDa from approximately amino acids 104 to 194. The Ana40 monoclonal reacts with amino acids 112 to 124, and this epitope is present in the trypsin-resistant fragment. The question remains how the endogenous BNIP3 remains in an inactive, nonintegrated state. At least two non-mutually exclusive mechanisms are possible: (i) endogenous BNIP3 assumes a conformation that prevents integration of the TM domain until it is altered by some posttranslational modification, or (ii) endogenous BNIP3 interacts with a regulatory protein that maintains it in an unintegrated form at the surface of the mitochondria until it dissociates. Since overexpression induces cell death, BNIP3 is able to overcome this inhibition in high concentrations, suggesting that the regulatory mechanism is saturable. Translocation from the cytoplasm to the mitochondria

during induction of apoptosis has been reported for several members of the BCL-2 proapoptotic family, including BID (Li, H. et al, 1998, Luo, X. et al, 1998), BAX (Goping, I. S. et al, 1998), BAK (Griffiths, G. J. et al, 1999), BAD (Datta, S. R. et al, 1997, 64), and BIM (Salvesen, G.S., et al., 1999). These molecules can be regulated by phosphorylation, dimerization, or proteolytic cleavage (Gross, A. et al, 1999). In the absence of an apoptotic stimulus, BAD is phosphorylated by Akt (64) and by mitochondrion-anchored protein kinase A (Harada, H. et al, 1999) and sequestered in the cytoplasm by 14-3-3 protein (64). BAX, BAK, and BIM are held inactive in the cytoplasm and are translocated to the mitochondria after a cell death signal. Further regulation is suspected for BAX, which is permitted to integrate into the mitochondrial mem-brane following proteolytic cleavage of an inhibitory element in the N terminus (Goping, I. S. et al, 1998). Similarly, BID is cleaved by caspase 8 following Fas ligation, resulting in mitochondrial translocation (Li, H. et al, 1998, Luo, X. et al, 1998). Whether endogenous BNIP3 translocation to the mitochondrial membrane is regulated by a posttranslational mechanism similar to these proteins remains to be determined. In conclusion, BNIP3 overexpression initiates a cell death pathway that is activated by protein integration into the outer mitochondrial membrane. This pathway requires PT pore opening and is independent of caspases, Apaf-1, and cytochrome *c* release. Cell death manifests as mitochondrial dysfunction, plasma membrane damage, and the morphology of necrosis.

Example 2

Neuronal cell death has been shown to occur by apoptotic, necrotic and alternative cell death mechanisms. Recently, it was reported that the gene BNIP3, a member of the Bcl-2 family without a functional BH3 domain, induces cell death in a necrotic-like manner and that this gene-regulated cell death pathway involves opening of the mitochondrial permeability transition pore without caspase activation and cytochrome *c* release; BNIP3-mediated cell death was induced by hypoxic challenge in non-neuronal cells. BNIP3 is a gene that when activated in brain is capable of inducing necrotic (possibly paraptotic) cell death. In rat brain, BNIP3 staining was not detectable by immunohistochemistry. However, BNIP3 expression was increased dramatically in models of brain injury and excitotoxicity. After blunt trauma to brain or intra-striatal injection of kainic acid, BNIP3 immunopositive cells

were observed adjacent to the sites of injury and the BNIP3 positive cells showed DNA fragmentation as demonstrated by TUNEL and Hoechst 33342 staining. Western blot analysis showed a 30 kDa band from kainic acid injected brain; positive immunoblot was blocked by a BNIP3-GST recombinant protein. Similar results were observed using a chronic seizure model using i.p. injections of kainic acid. *In situ* hybridization results using a RNA riboprobe revealed that intrastriatal injections of kainic acid increased levels of BNIP3 mRNA in sites of brain injury. These results show that BNIP3 is a new target for neuronal rescue strategies.

Example 3

Neuronal cell death has been shown to occur by apoptotic, necrotic, and alternative cell death mechanisms. Recently, it was reported that BNIP3, a member of the Bcl-2 protein family without a functional BH3 domain, induces cell death in a necrotic-like manner and that this gene-regulated cell death pathway involves opening of the mitochondrial permeability transition pore without caspase activation and cytochrome c release. However, to date nothing is know about the role of BNIP3 in neuronal cell death. BNIP3, when expressed in neurons, is capable of inducing necrotic (possibly parapoptotic) cell death. Glutamate was applied to cultures of rat hippocampal neurons, expression of BNIP3 was detected using immunohistochemical techniques, and cell death was measured morphologically and by using trypan blue exclusion. BNIP3 expression following application of glutamate was both time- and glutamate concentration-dependent. Cell death was also found to be time- and concentration-dependant; increased numbers of trypan blue positive cells paralleled the increased number of cells immunopositive for BNIP3. Expression of BNIP3 was seen in those cells with morphological features associated with necrotic rather than apoptotic cell death. BNIP3 is involved in excitotoxic cell death and is a mediator of parapoptotic neuronal cell death.

Example 4

Cardiac cell death has been shown to occur by apoptotic, necrotic and alternative cell death mechanisms. Recently, it was reported that the gene BNIP3, a member of the Bcl-2 family without a functional BH3 domain, induces cell death in a necrotic-like manner and that this gene-regulated cell death pathway involves

opening of the mitochondrial permeability transition pore without caspase activation and cytochrome c release; BNIP3-mediated cell death was induced during by hypoxic challenge in non-cardiac cells. BNIP3 is a gene when activated in heart is capable of inducing necrotic (possibly paraptotic) cell death. In the rat heart, BNIP3 staining was not detectable by immunocytochemistry. However, BNIP3 expression was increased markedly in models of heart injury. After myocardial infarction induced by coronary artery blood flow insufficiency, BNIP3 positive staining was detected in cells in the peri-infarct zone including scare tissue and in cells adjacent to primary site of injury (necrotic core). Western Blot analysis showed monomeric and homodimeric bands at 30kDa and 60kDa respectively. BNIP staining cells displayed dismorphoric nuclei and DNA structure by Hoechst 33258 staining. These studies indicate that BNIP3 is a new target for cardiac cell rescue strategies.

Example 5

Cardiac cell death has been shown to occur by apoptotic, necrotic and alternative cell death mechanisms. Recently, it was reported that the gene BNIP3, a member of the Bcl-2 family without a functional BH3 domain, induces cell death in a necrotic-like manner and that this gene-regulated cell death pathway involves opening of the mitochondrial permeability transition pore without caspase activation and cytochrome c release; BNIP3-mediated cell death was induced during by hypoxic challenge in non-cardiac cells. BNIP3 is a gene when activated in heart is capable of inducing necrotic (possibly paraptotic) cell death. In rat cardiac myocytes BNIP3 expression was not detectable. BNIP3 was localized to mitochondrial membrane under normoxic conditions. However, BNIP3 expression was increased dramatically in cardiac myocytes subjected to hypoxic challenge. BNIP3 protein mitochondrial and sub-cellular membrane factions. Western Blot analysis showed monomeric and homodimeric bands at 30kDa and 60kDa respectively. Cardiac myocyte death by Hoechst 33258 staining was increased in cells during hypoxia along with BNIP3 expression. These studies indicate that BNIP3 is a new target for cardiac cell rescue strategies.

Example 6

Cardiac cell death has been shown to occur by apoptotic, necrotic and alternative cell death mechanisms. Recently, it was reported that the gene BNIP3, a member of the Bcl-2 family without a functional BH3 domain, induces cell death in a necrotic-like manner and that this gene-regulated cell death pathway involves opening of the mitochondrial permeability transition pore without caspase activation and cytochrome c release; BNIP3-mediated cell death was induced during by hypoxic challenge in non-cardiac cells. BNIP3 is a gene when activated in heart is capable of inducing necrotic (possibly paraptotic) cell death. In rat cardiac myocytes BNIP3 expression was not detectable. BNIP3 was localized to mitochondrial membrane under normoxic conditions. However, BNIP3 expression was increased dramatically in cardiac myocytes subjected to hypoxic challenge. BNIP3 protein was integrated to mitochondrial and sub-cellular membrane factions during hypoxia. Western Blot analysis showed monomeric and homodimeric bands at 30kDa and 60kDa respectively. Adenovirus mediated gene transfer of a viral vector designed to express mutant for of BNIP3 (DN BNIP3 protein) was delivered to cardiac myocytes with uniformity and high efficiency. Mitochondrial defects including changes to mitochondrial membrane potential and permeability transition pore opening associated with hypoxic injury were observed in cardiac myocytes. Nuclear defects associated with changes to DNA structure were observed in hypoxic cardiac myocytes by Hoechst 33258 staining. However, mitochondrial defects in cardiac myocytes subjected to hypoxia were suppressed in cardiac myocytes genetically modified to express the DN BNIP3 protein. Nuclear defects in cardiac myocytes were suppressed in cells expressing the DN BNIP3 protein. Expression of the DN BNIP3 protein suppressed morphological features of cardiac myocyte cell death during hypoxia as determined by calcein acteoxy-methyl ester (AM) and ethidium homodimer staining. These studies indicate that BNIP3 is a mediator of apoptotic and necrotic cell death of cardiac myocytes. BNIP3 is a new target for cardiac cell rescue strategies.

METHODS

Recombinant Adenovirus

The AdBNIP3 CrmA adenovirus, consisting of the wild type and mutant DN BNIP3 cDNA, was generated by homologous recombination in human embryonic 293 kidney cells, by a method that has previously been reported (Kirshenbaum, LA et al, 1993)[14]. Twenty-four hours after myocyte isolation, cells were infected in serum-free DMEM with AdCrmA virus at a titer of 20 plaque-forming units per cell for 4-6 hours. The viral suspension was removed and cells were incubated for an additional 20 hours in supplement DMEM prior to experimentation (Kirshenbaum, LA et al., 1997).

Hypoxia

Post-natal ventricular myocytes were subjected to hypoxia for 24 hours in an air-tight chamber in culture media that was continually gassed with 95% N_2-5% CO_2. These conditions were modeled after preliminary studies (de Moissac, D et al., 2000; Bialik ,S et al, 1999) demonstrating that this duration of hypoxia was sufficient to trigger apoptosis of neonatal ventricular myocytes.

Assays of apoptosis

Genomic DNA was subjected to gel electrophoresis as previously reported (Bialik, S et al, 1999). Nuclear morphology was assessed by Hoechst 33258 dye (Molecular Probes, Eugene Oregon) as reported[15]. Cells were visualized using a Olympus AX70 epifluorescence microscope as described previously (de Moissac, D et al., 2000)[4;15].

Immunofluorescence Microscopy

Following interventions, myocytes were incubated with 0.1μM MitoTracker Red, (chloromethyl-rosamine CMX-Ros, Molecular Probes, Eugene Oregon) for the detection of intact respiring mitochondria. Fixed cells were incubated with 1μg/ml of a murine antibody directed toward cytochrome c (Pharmingen) followed by anti-mouse conjugated fluorescein IgG (1:150) (Roche Diagnostics) and detected using an Olympus Fluoview confocal microscope[4].

Western Blot Analysis

For detection of BNIP3 proteins the Western Blot filter was probed with a rabbit antibody directed toward BNIP3 proteins. The cytoplasmic S-100 fraction was prepared by

methods previously described [4;17] Bialik, S et al, 1999). Appropriate control experiments were employed to ensure purity and completeness of separation of mitochondrial and S-100 fractions [4]. Bound proteins were visualized using enhanced chemiluminesence (ECL) reagents (Amersham).

Mitochondrial membrane potential $\Delta\Psi_m$ and MPT

Mitochondrial membrane potential $\Delta\Psi_m$, was monitored using the potential sensitive dyes JC-1 dye (1μM, 5,5',6,6'-tetraethylbenzimidazolylcarbocyanine iodide) or TMRM (50nM, tetramethyl rhodamine methyl ester perchlorate, Molecular Probes, Eugene Oregon). A fluorescence distribution curve of individual cells was generated for each condition tested. Fluorescent intensities were then compared to each respective control group. To monitor mitochondrial permeability transition (PT), ventricular myocytes were loaded with 5μM calcein-acetoxymethylester (calcein-AM, Molecular Probes, Eugene Oregon) in the presence of 2-5mM cobalt chloride to quench the cytoplasmic signal (Petronilli,V, et al., 1998).

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

BNIP3 constructs and deletion mutants generated in the lab	
Construct	**Mutation (amino acid residues)**
BNIP3	None
BNIP3DBH3	D104-119
BNIP3DN;BH3	D1-49;D104-119
BNIP3DC	D185-194
BNIP3DCD	D112-130
BNIP3DN	D1-49
BNIP3D50-194	D50-194
BNIP3DN;DTM	D1-49;D164-194
BNIP3DTM1	D164-184
BNIP3DTM2	D164-194
BNIP3D1-12	D1-12
BNIP3D1-100	D1-100
BNIP3D1-124	D1-124
BNIP3D41-100	D41-100
BNIP3D49-151	D49-151
BNIP3D41-151	D41-151
BNIP3D58-66	D58-66
BNIP3D120-130	D120-130
BNIP3D104-130	D104-130
BNIP3D112-119	D112-119
BNIP3D65-151	D65-151
BNIP3D173-194	D173-194
BNIP3D179-184	D179-184
BNIP3D162-170	D162-170
BNIP3D183-184	D183-184
BNIP3D181-184	D181-184
BNIP3D179-194	D179-184
BNIP3D190-194	D190-194
BNIP3(P167 to S)	P167 to S
BNIP3(L179 to S)	L179 to S
BNIP3(G180 to T)	G180 to T
BNIP3(G180 to E)	G180 to E
BNIP3(A176 to V; G180 to E)	A176 to V; G180 to E
BNIP3(L179 to T; G180 to E)	L179 to T; G180 to E
BNIP3-BclTM	BNIP3(1-163) + BclTM (219-239)
BNIP3-CybTM	BNIP3(1-163) + Cytob5 (100-134)
* NOTE: Many of these BNIP3 deletion mutants are cloned into yeast (pAS1, pACTII, pGBT9) vectors as well as mammalian expression vector pcDNA3. Furthermore pcDNA3 clones may have a 3' or 5' HA- or T7 epitote tag	

BNIP3 Wild type promoter (Seq ID No: 1)

ctttcccgcaag
accagacacg actgtctggg aagcagcgtt tctggggcgc accttgacac ttggatttgg
atcaacaatg ctttcaagaa gaaagacttt tgatcaaaag cgggaaatga gaaagcgact
t tcctctgaa aagtgcctcc cagtcccgag gctgcgaggc ccccacgcca ggctggctcc
cacggaagcc gggcacccac ccggaccgac caagcgccac tccgccccgt ggacggggcg
tcccaccccg gggacgcccg ccccacaccg cgtttgcacc ccggaggccc cttgccgcag
aggcggacgg cgcgcctctc ccgggccccc ggggtccgcg cctccctcgg gcagactctt
tcgactctgc tcgagcctcc gcttcttcct gcgggcggac gccccggaca caacgggccc
cgctgttcac gcaggggcgc cccggcgggg cgggcaaaga cccggggacg cggtcccgtc
ccgagacgct cagctccggc ccaccgctcg cagctcccgc cccgggcgca ggtcccgacc
ccacgggccg tctcggagcc gcagcggccg cttcctgca cgtcctcacg cccccgcac
ggacgccgcc agccccgcgc ctcagtttcc ccactagcag gatggaaaga cgggccccgc
cccgaagcgt agcggcgtct ccgtggtagc cagtgcccag agagtccgcc ggtcccaccg
cccattcaaa ggagaacccg gcccaccgcc cgccgcggcg gcgaccgcgc agcccactcg
tcacgcggcc cgcggcgtcc agcccgggcc ggctcacctc aggcggtcgc tgccgccctc
gcgcctgcgc gcccctcgcc ccgcccctct ccccgcccgc gtcccgcgca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgcccctg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP3 D1 : Deletion mutant 1 (Seq ID No: 2)

tcctctgaa aagtgcctcc cagtcccgag gctgcgaggc ccccacgcca ggctggctcc
cacggaagcc gggcacccac ccggaccgac caagcgccac tccgccccgt ggacggggcg
tcccaccccg gggacgcccg ccccacaccg cgtttgcacc ccggaggccc cttgccgcag
aggcggacgg cgcgcctctc ccgggccccc ggggtccgcg cctccctcgg gcag actctt
tcgactctgc tcgagcctcc gcttcttcct gcgggcggac gccccggaca caacgggccc
cgctgttcac gcaggggcgc cccggcgggg cgggcaaaga cccggggacg cggtcccgtc
ccgagacgct cagctccggc ccaccgctcg cagctcccgc cccgggcgca ggtcccgacc
ccacgggccg tctcggagcc gcagcggccg cttcctgca cgtcctcacg cccccgcac
ggacgccgcc agccccgcgc ctcagtttcc ccactagcag gatggaaaga cgggccccgc
cccgaagcgt agcggcgtct ccgtggtagc cagtgcccag agagtccgcc ggtcccaccg
cccattcaaa ggagaacccg gcccaccgcc cgccgcggcg gcgaccgcgc agcccactcg
tcacgcggcc cgcggcgtcc agcccgggcc ggctcacctc aggcggtcgc tgccgccctc
gcgcctgcgc gcccctcgcc ccgcccctct ccccgcccgc gtcccgcgca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgcccctg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP3 D2 : Deletion mutant 2 (Seq ID No: 3)

actctt
tcgactctgc tcgagcctcc gcttcttcct gcgggcggac gccccggaca caacgggccc
cgctgttcac gcaggggcgc cccggcgggg cgggcaaaga cccggggacg cggtcccgtc
ccga gacgct cagctccggc ccaccgctcg cagctcccgc cccgggcgca ggtcccgacc
ccacgggccg tctcggagcc gcagcggccg cttcctgca cgtcctcacg cccccgcac
ggacgccgcc agccccgcgc ctcagtttcc ccactagcag gatggaaaga cgggccccgc
cccgaagcgt agcggcgtct ccgtggtagc cagtgcccag agagtccgcc ggtcccaccg
cccattcaaa ggagaacccg gcccaccgcc cgccgcggcg gcgaccgcgc agcccactcg
tcacgcggcc cgcggcgtcc agcccgggcc ggctcacctc aggcggtcgc tgccgccctc
gcgcctgcgc gcccctcgcc ccgcccctct ccccgcccgc gtcccgcgca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgcccctg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc

cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP 3 D3 : deletion mutant 3 (Seq ID No: 4)

gacgct cagctccggc ccaccgctcg cagctcccgc cccgggcgca ggtcccgacc
ccacgggccg tctcggagcc gcagcggccg cttcctgca cgtcctcacg cccccgcac
ggacgccgcc agccccgcgc ctcagtttcc ccactagcag gatggaaaga cgggccccgc
cccgaagcgt agcggcgtct ccgtggtagc cagtgcccag agagtccgcc ggtcccaccg
ccccttcaaa ggagaacccg gcccaccgcc cgccgcggcg gcgaccgcgc agcccactcg
tcacgcggcc cgcggcgtcc agcccgggcc ggctcacctc aggcggtcgc tgccgccctc
gcgcctgcgc gcccctcgcc ccgcccctct ccccgcccgc gtcccgcgca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgccccg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP3 D4 : deletion mutant 4 (Seq ID No: 5)

gtggtagc cagtgcccag agagtccgcc ggtcccaccg
ccccttcaaa ggagaacccg gcccaccgcc cgccgcggcg gcgaccgcgc agcccactcg
tcacgcggcc cgcggcgtcc agcccgggcc ggctcacctc aggcggtcgc tgccgccctc
gcgcctgcgc gcccctcgcc ccgcccctct ccccgcccgc gtcccgcgca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgccccg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP3 D5 : Deletion mutant 5 (Seq ID No: 6)

ca ccggaggcct
ctgcccctcg cccaccgcag gacccgcccc gcgcacgcgc cgcacgtgcc acacgcaccc
cacgccccg cgcacgcgca ggccccaagt cgcggccaat gggcgacgcg gccgcagatc
cgcccggccc cgccctgccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

BNIP3 D6 : Deletion mutant 6 (Seq ID No: 7)

gccc tgtgagttcc tccggccggg ctgcggggct ccgctcagtc
cgggagcgca gctgggccgc ggcgctccga cctccgcttt cccaccgccc gcagctgaag
cacatcccgc agcccggcgc ggactccgat cgccgcagtt g

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CLAIMS

What is claimed is:

1. An isolated and purified BNIP3 protein.

2. The protein according to Claim 1, wherein said protein induces necrosis in cells.

3. The protein according to Claim 1, wherein said protein induces necrosis in cancer cells.

4. The protein according to Claim 1, wherein said protein including a sequence selected from the group consisting essentially of Seq ID No. 1-7.

5. A method of inducing necrosis in cells by transfecting a cell with a BNIP3 protein.

6. The method according to Claim 5, wherein said transfecting step is further defined as transfecting into a cancer cell.

7. The method according to Claim 5, wherein said transfecting step further includes transfecting using a vector.

8. The method according to Claim 4, wherein said transfecting step further includes transfecting using raw DNA.

9. A method of inducing necrosis in cells by creating a transgene overexpressing the gene for the BNIP3 protein.

10. The method according to Claim 9, wherein said creating step further creates a transgene for inducing necrosis in cancer cells.

11. A transgenic animal and progeny thereof having an expression vector consisting essentially of a BNIP3 protein selected from the group consisting essentially of Seq. ID No. 1-7.

12. A method of preventing necrosis in cells by controlling expression of a BNIP3 protein.

13. The method according to claim 12, wherein said controlling step includes limiting the expression of the BNIP3 protein.

14. A method of protecting cells against cell death by administering an effective amount of DN NIP.

15. A vector for use in treating disease comprising BNIP3 and a promoter.

16. A vector for use in treating disease comprising DN NIP and a promoter.

17. A gene therapy comprising DNA encoding BNIP3 and a promoter, whereby said gene therapy is used to induce necrosis.

18. A gene therapy comprising DNA encoding DN NIP and a promoter, whereby said gene therapy is used to prevent cell death.

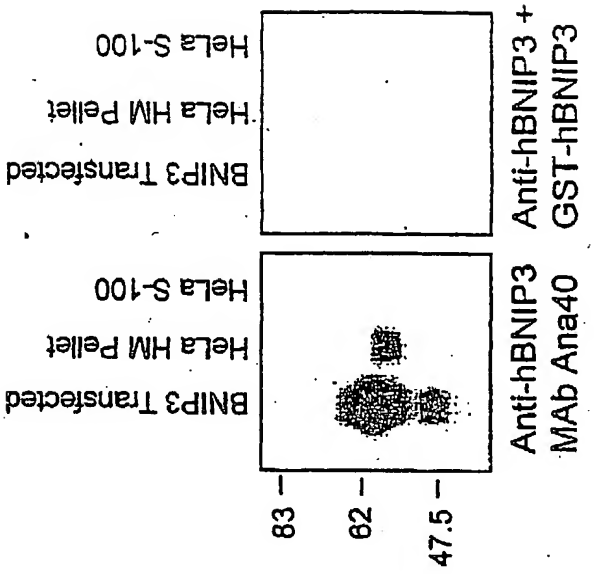


Figure 1A, right panel

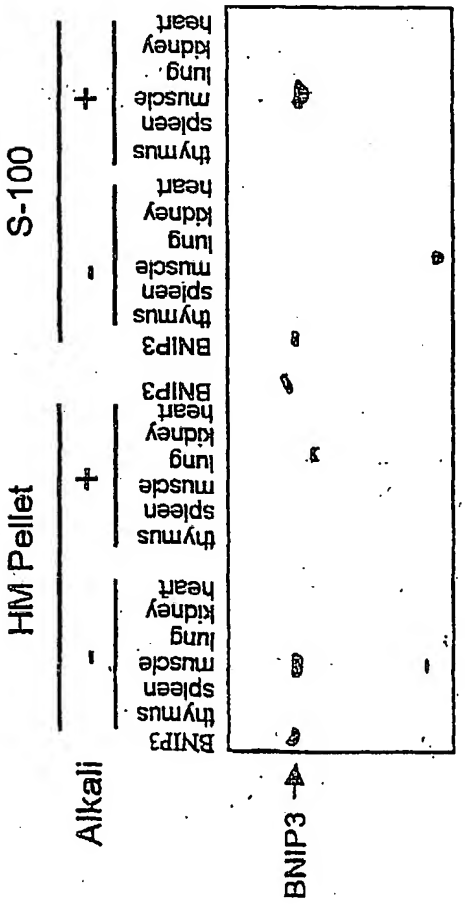


Figure 1A, left panel

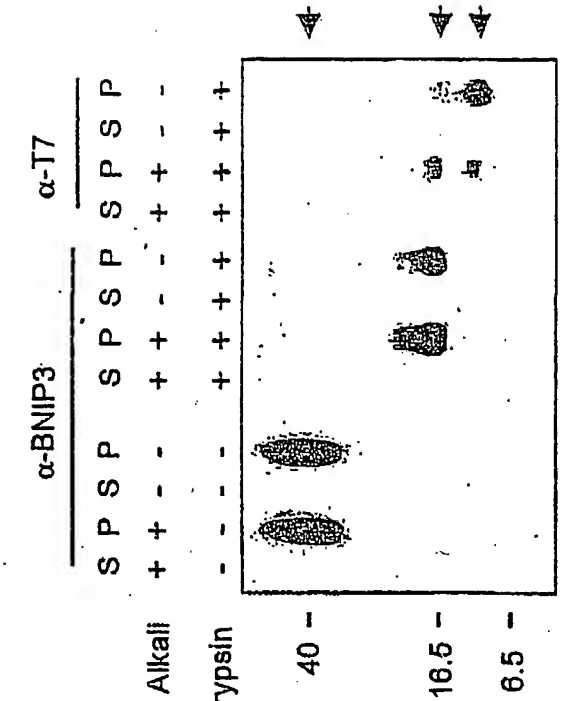


Figure 1C

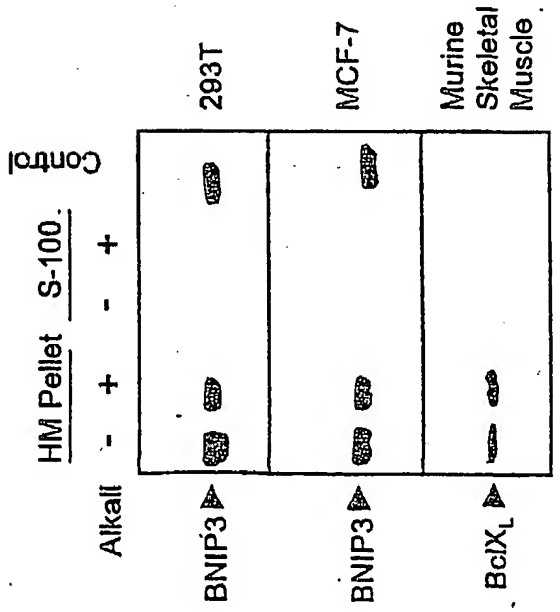


Figure 1B

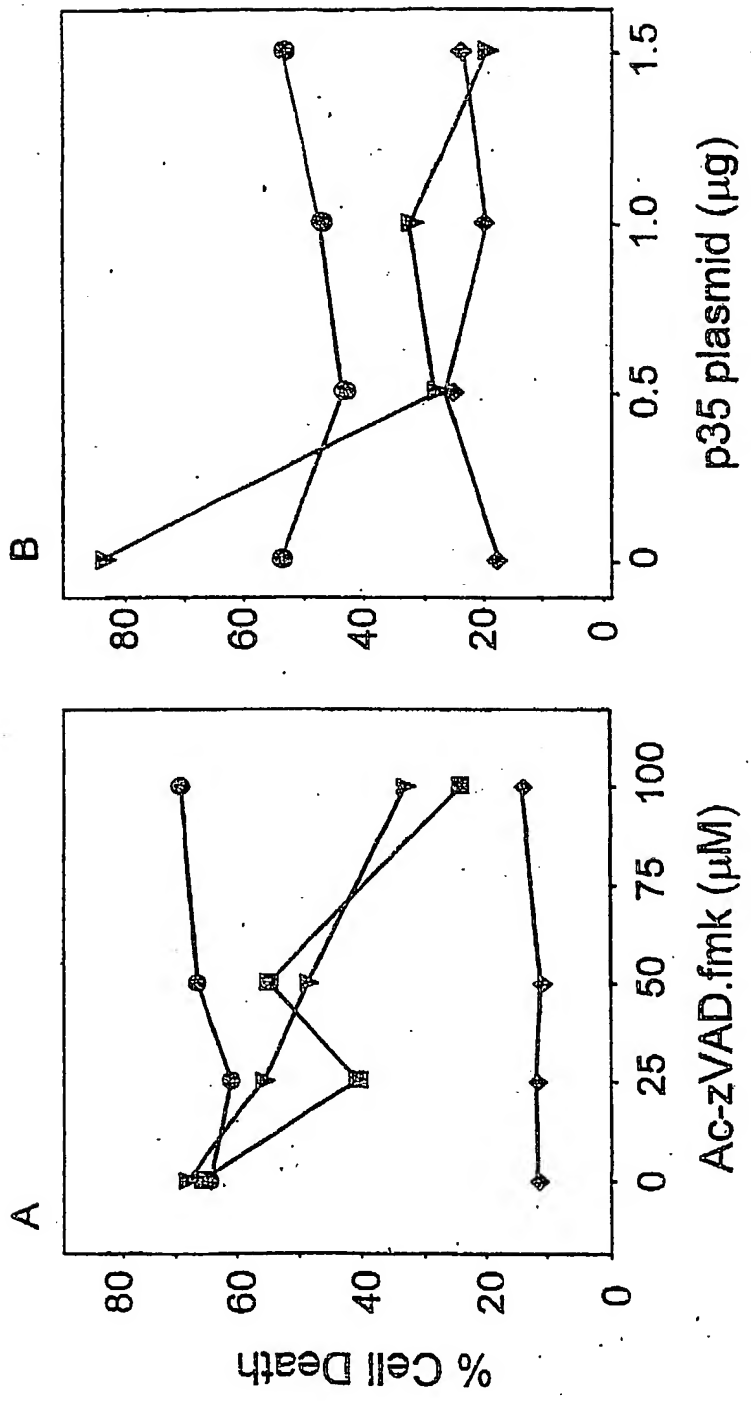

A
% Cell Death
0
20
40
60
80
0
25
50
75
100
Ac-zVAD.fmk (μM)
B
0
20
40
60
80
0
0.5
1.0
1.5
p35 plasmid (μg)

Figure 2A,B

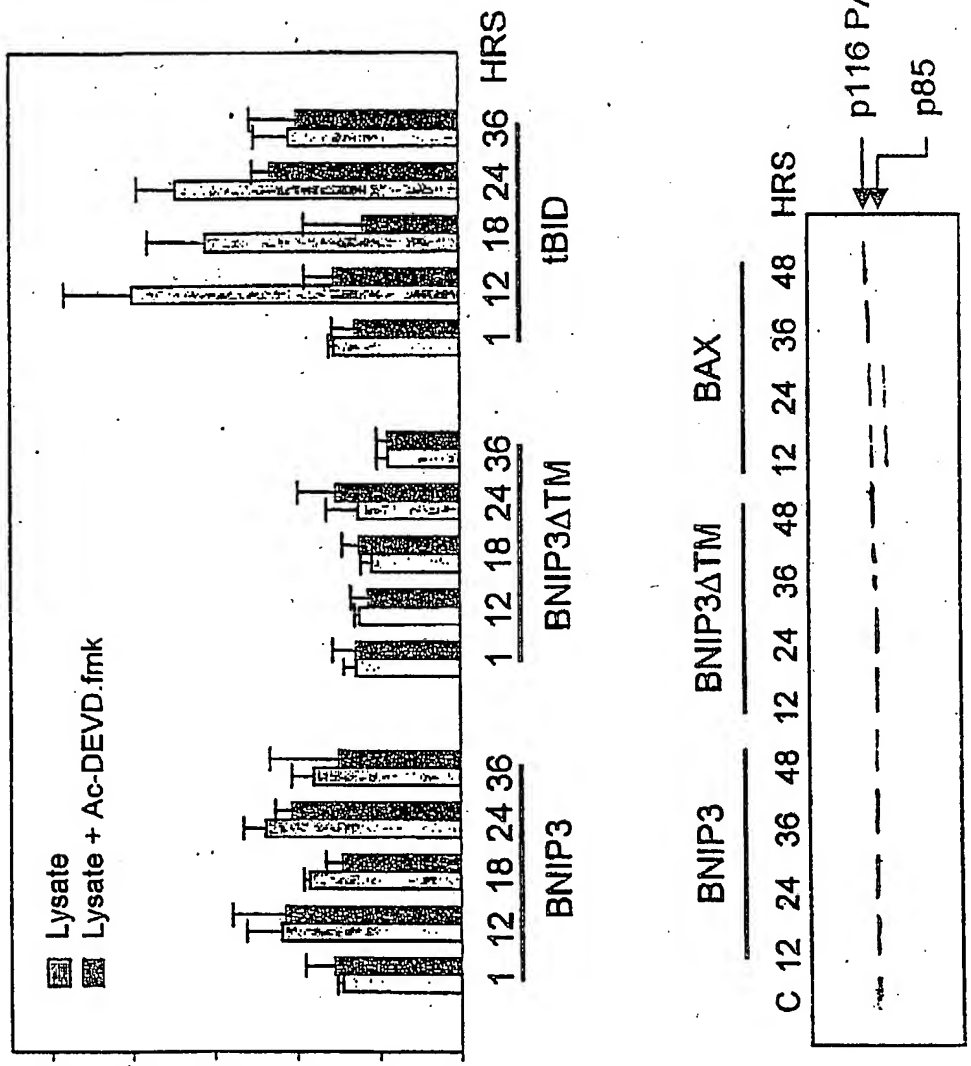

A
Fold Increase DEVDase ± SE
0
1
2
3
4
5
Lysate
Lysate + Ac-DEVD.fmk
1 12 18 24 36
BNIP3
1 12 18 24 36
BNIP3ΔTM
1 12 18 24 36 HRS
tBID
B
BNIP3
BNIP3ΔTM
BAX
C 12 24 36 48 12 24 36 48 12 24 36 48 HRS
p116 PARP
p85

Figure 3A,B

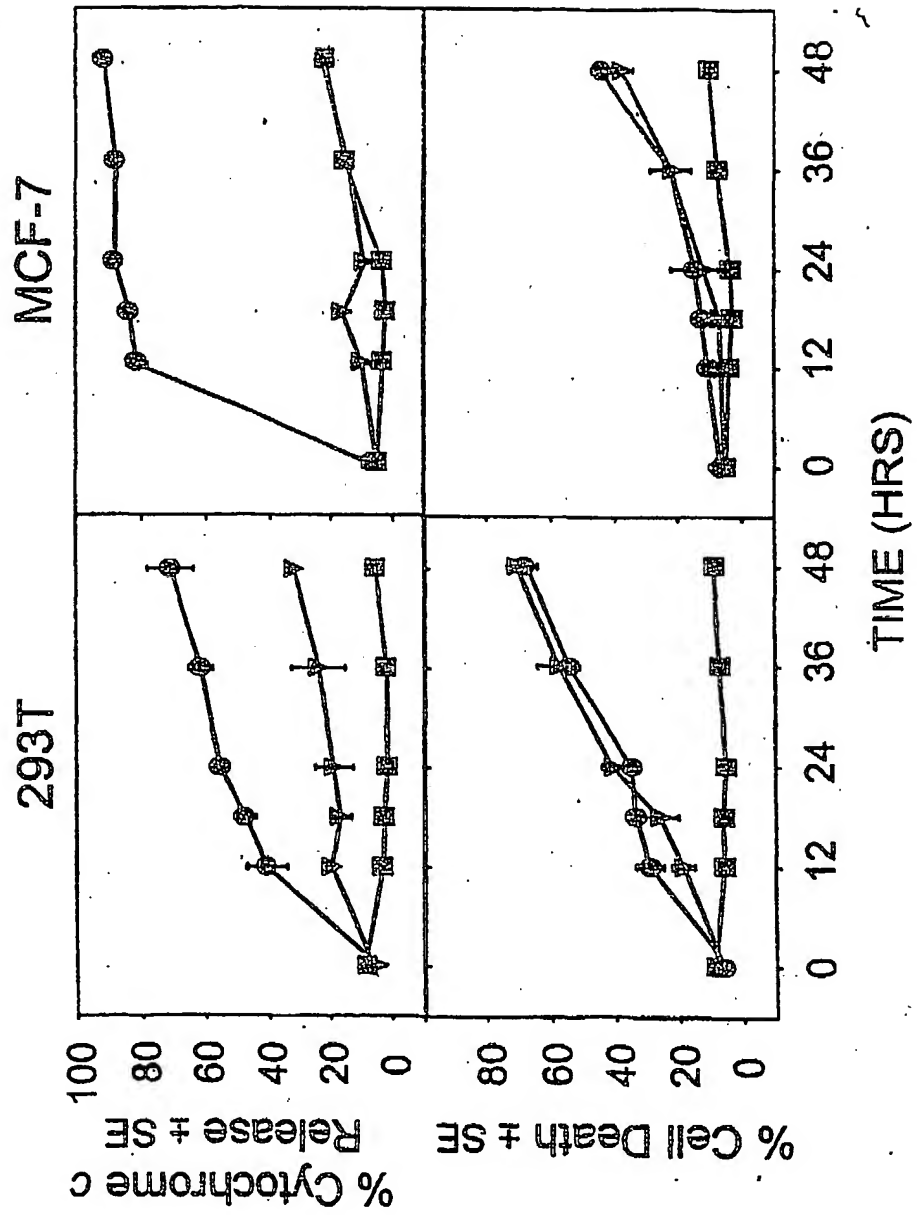

MCF-7
293T
% Cytochrome c Release ± SE
% Cell Death ± SE
100
80
60
40
20
0
0
12
24
36
48
TIME (HRS)

Figure 4A

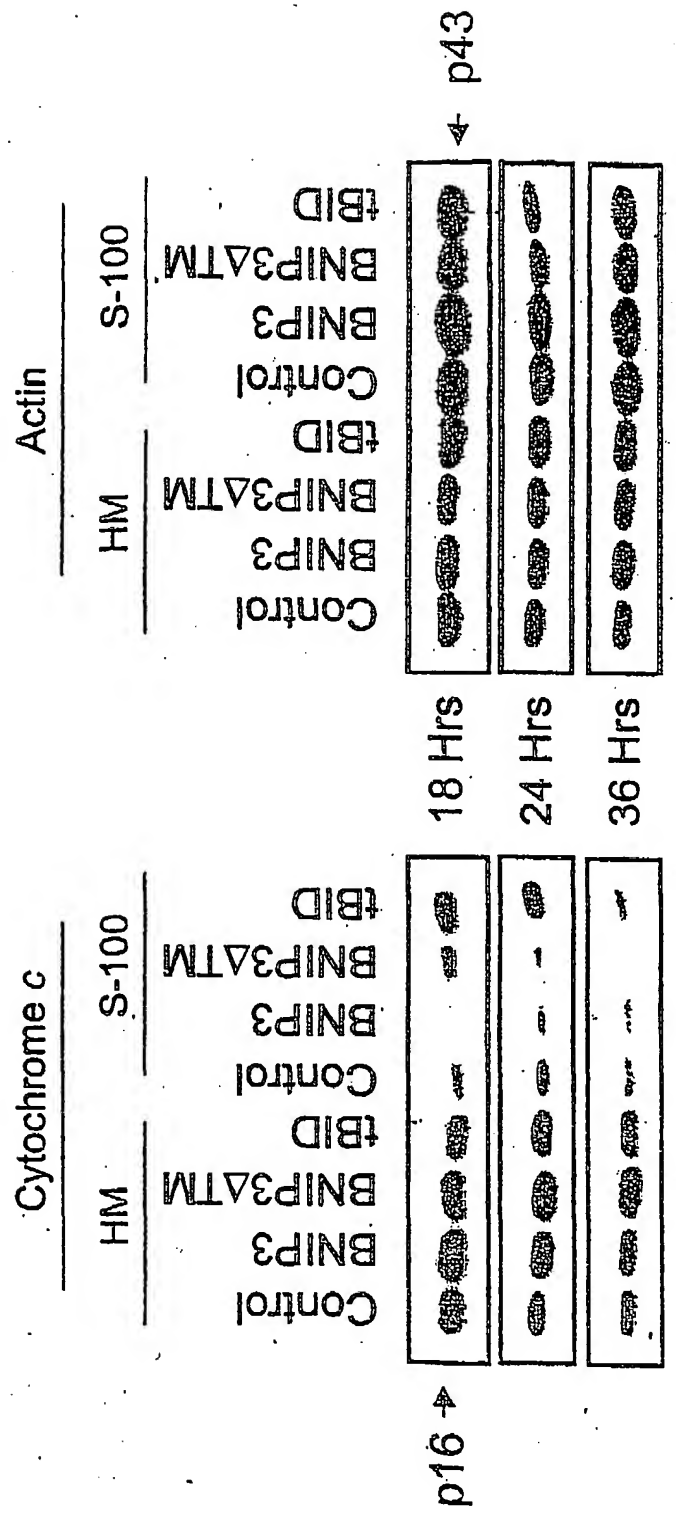

Cytochrome c
Actin
HM
S-100
Control
BNIP3
BNIP3ΔTM
tBID
p16
p43
18 Hrs
24 Hrs
36 Hrs

Figure 4B

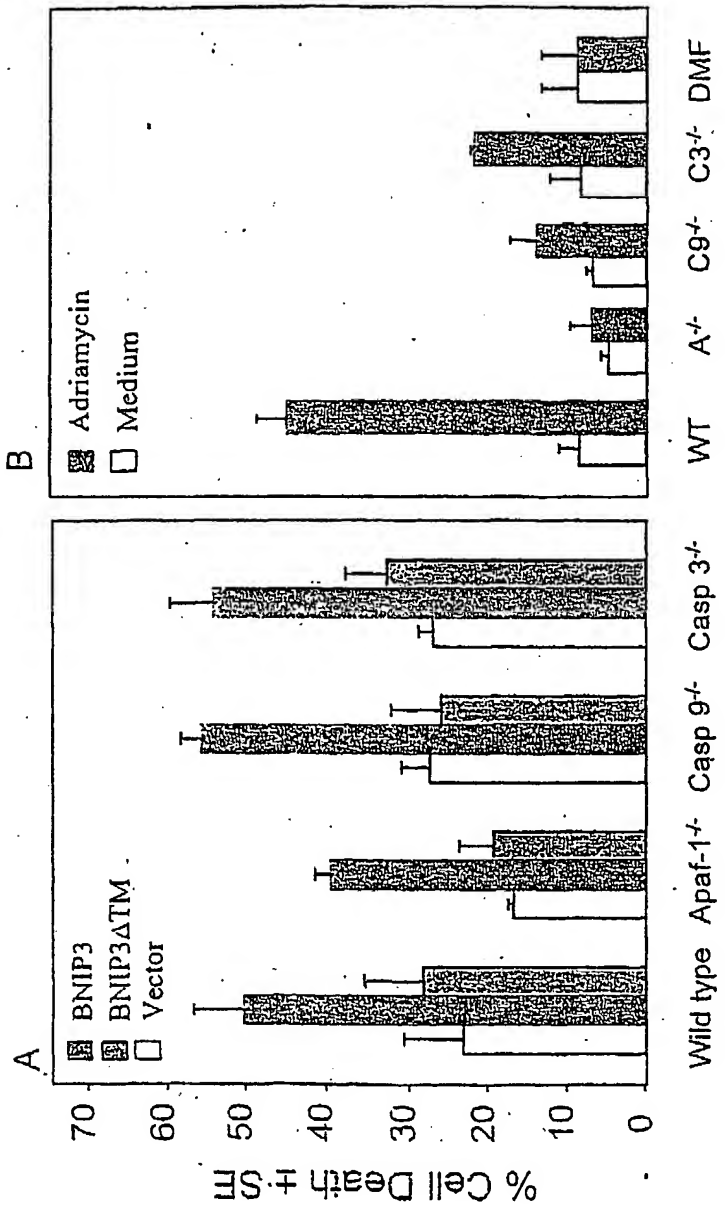

A
BNIP3
BNIP3ΔTM
Vector
% Cell Death +-SE
0
10
20
30
40
50
60
70
Wild type
Apaf-1-/-
Casp 9-/-
Casp 3-/-
B
Adriamycin
Medium
WT
A-/-
C9-/-
C3-/-
DMF

Figure 5A,B

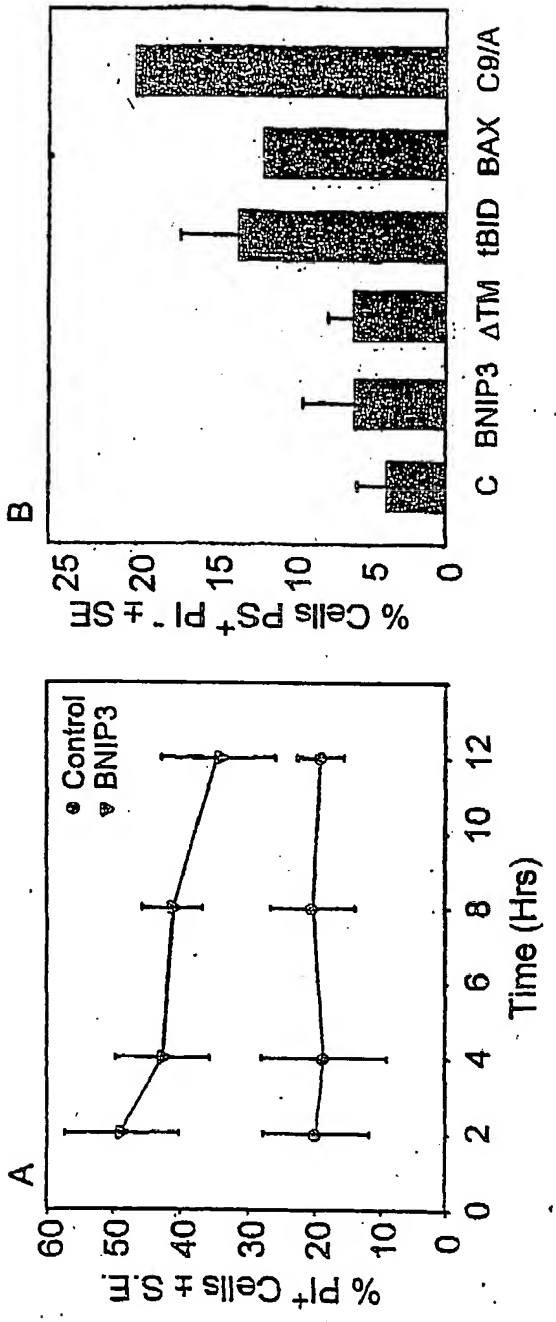

A
% PI+ Cells ± S.E.M.
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Time (Hrs)
Control
BNIP3
B
% Cells PS+ PI- ± SEM
25
20
15
10
5
0
C
BNIP3
ΔTM
tBID
BAX
C9/A

Figure 6 A,B

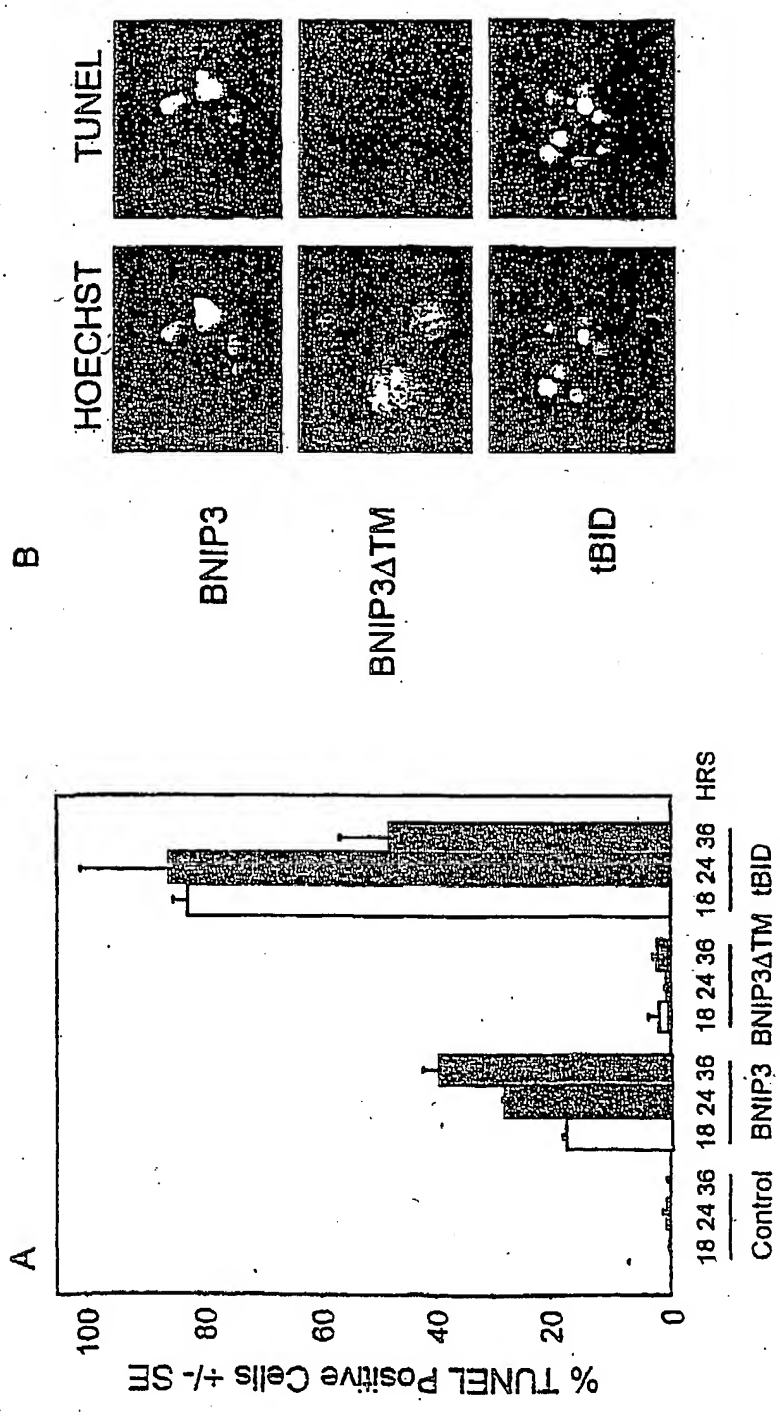

A
% TUNEL Positive Cells +/- SE
0
20
40
60
80
100
18 24 36 18 24 36 18 24 36 18 24 36 HRS
Control BNIP3 BNIP3ΔTM tBID
B
HOECHST
TUNEL
BNIP3
BNIP3ΔTM
tBID

Figure 7A,B

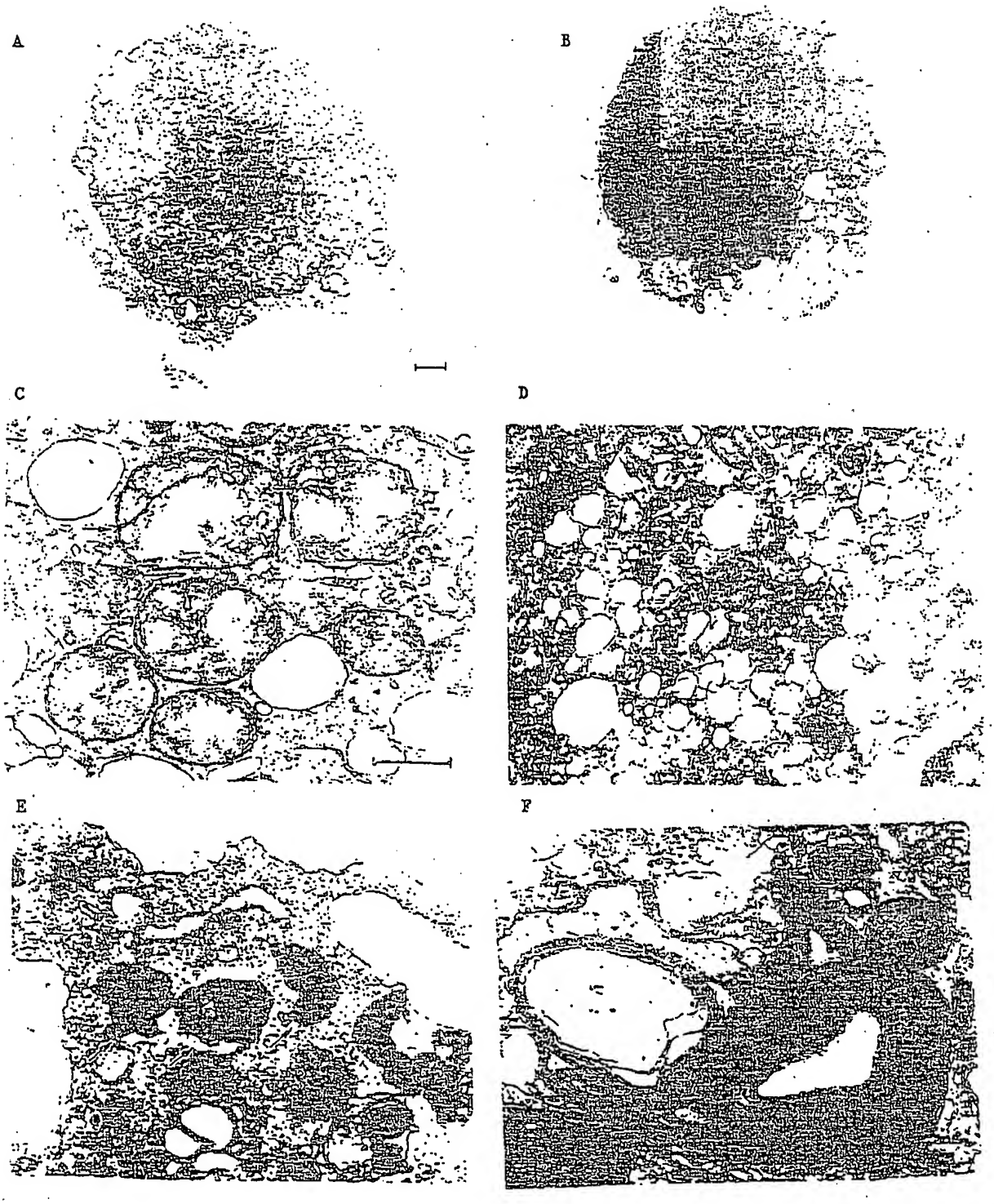


Figure 8

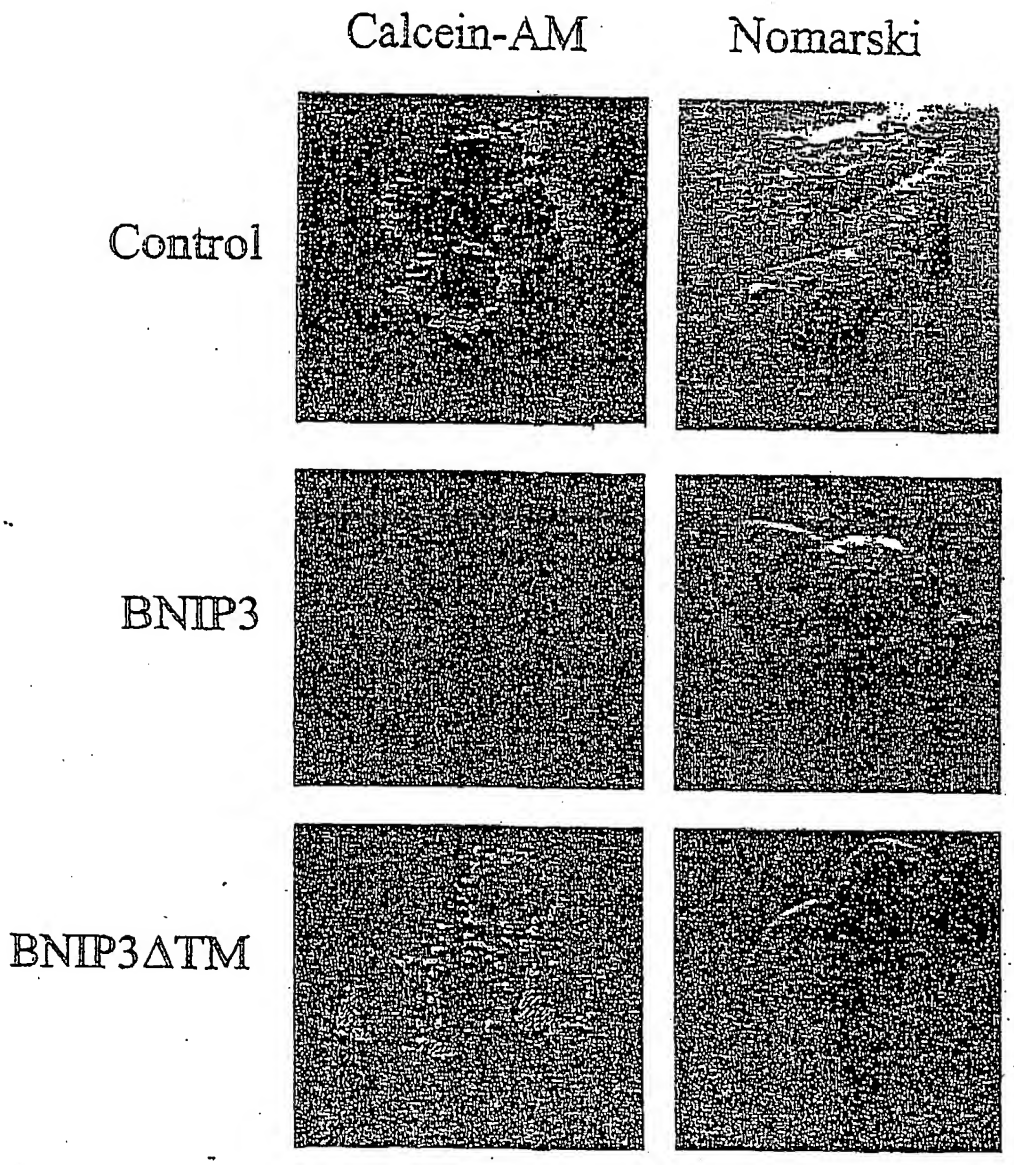


Figure 9A

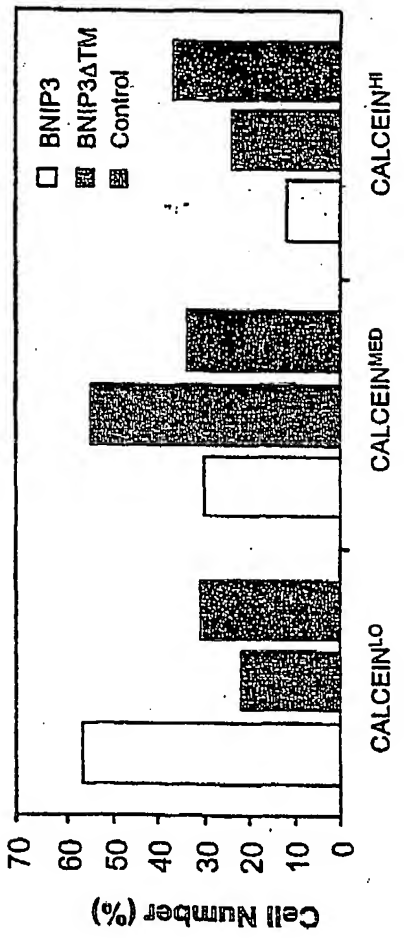

BNIP3
BNIP3ΔTM
Control
Cell Number (%)
0
10
20
30
40
50
60
70
CALCEIN^LO
CALCEIN^MED
CALCEIN^HI

Figure 9B

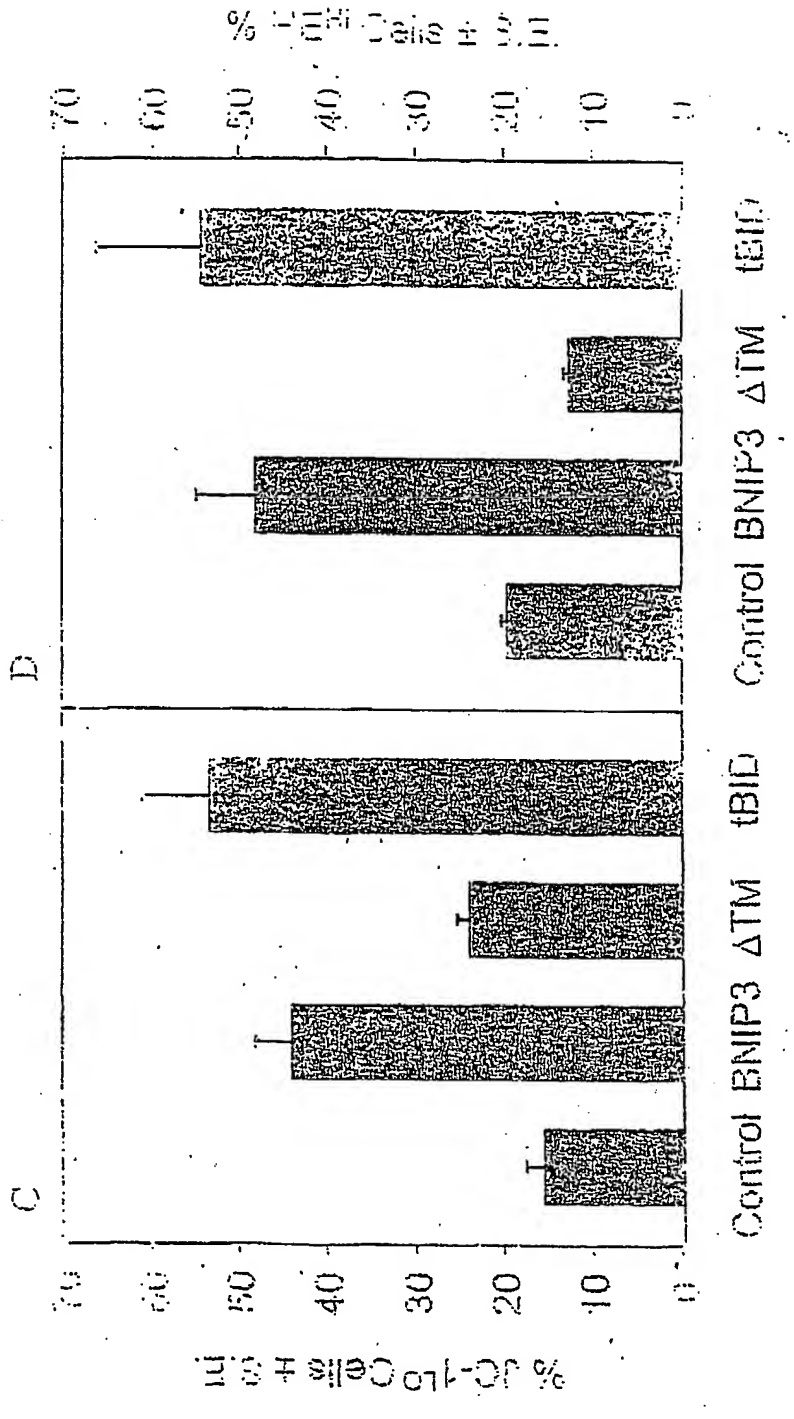


Figure 6 C,D

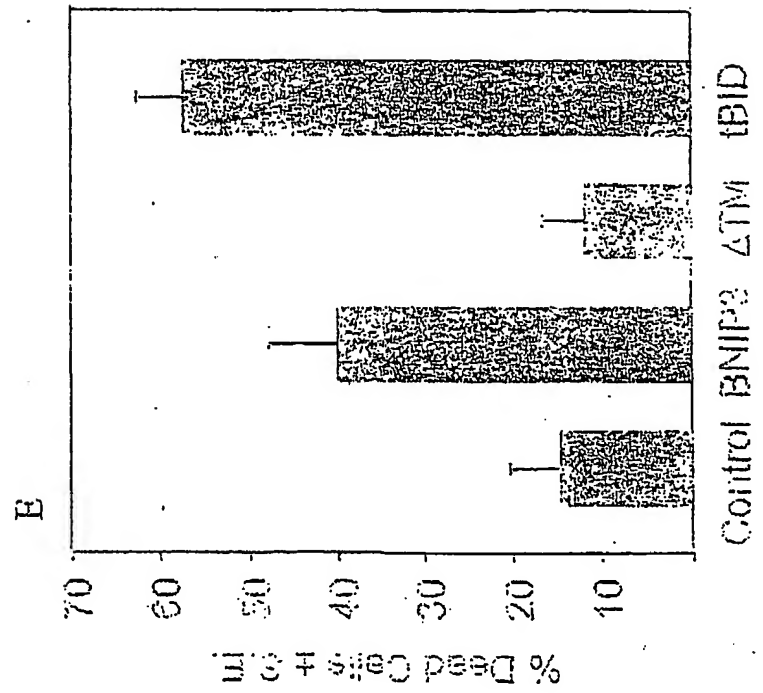


Figure 9E

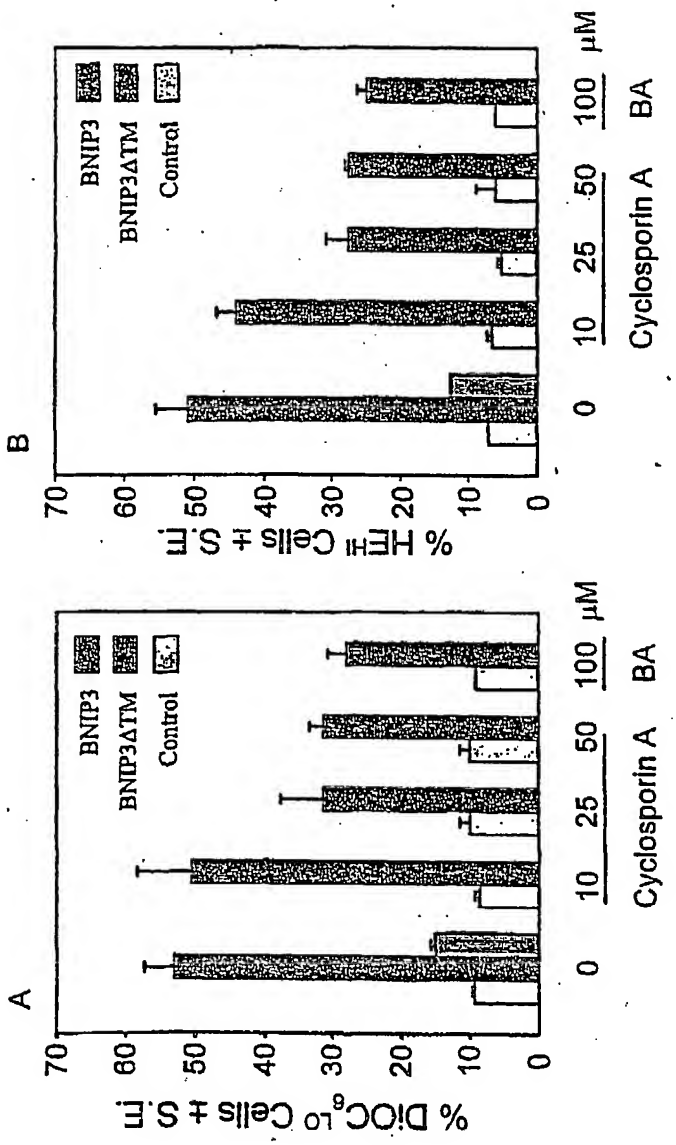

A
B
BNIP3
BNIP3ΔTM
Control
% DiOC6Lo Cells ± S.E.M.
% HEHI Cells ± S.E.M.
0
10
20
30
40
50
60
70
0
10
25
50
100 μM
Cyclosporin A
BA

Figure 10A,B

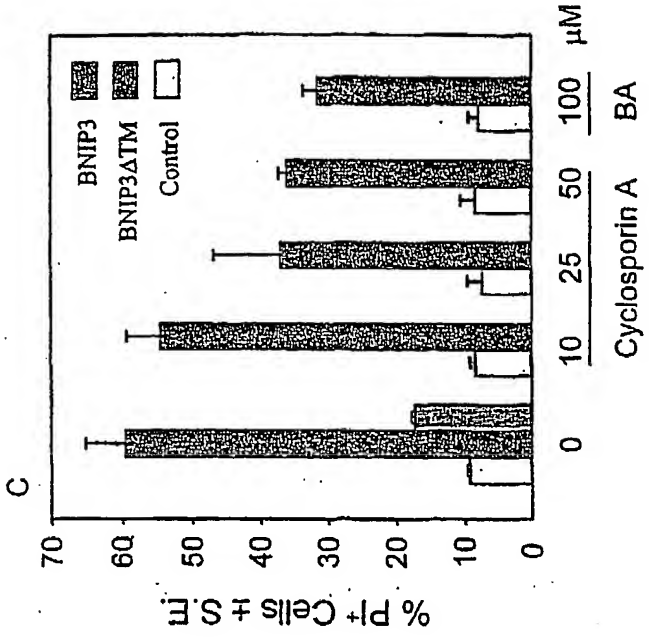

C
BNIP3
BNIP3ΔTM
Control
% PI+ Cells ± S.E.M.
70
60
50
40
30
20
10
0
0
10
25
50
100
μM
Cyclosporin A
BA

Figure 10C

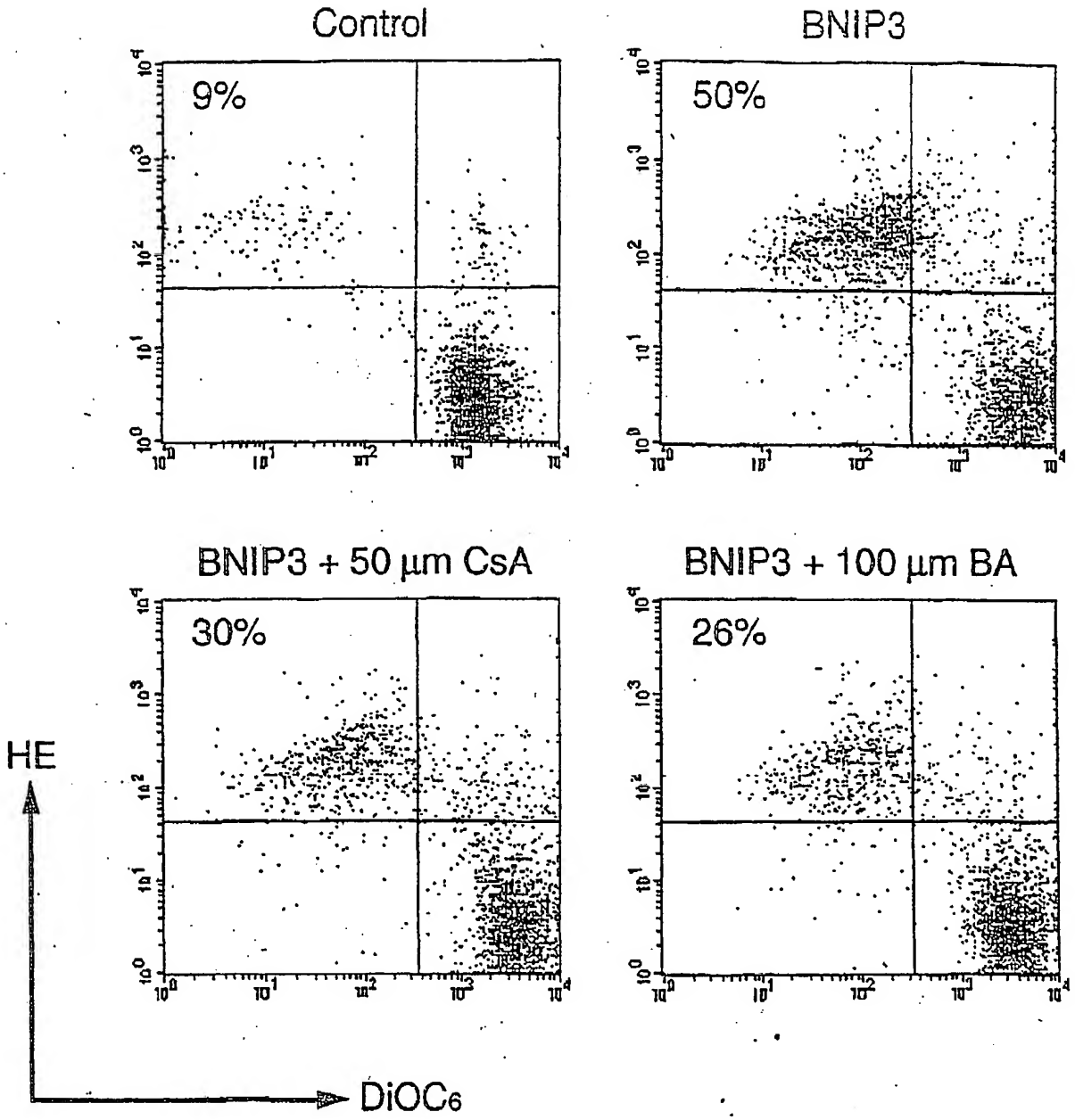

Control
9%
BNIP3
50%
BNIP3 + 50 µm CsA
30%
BNIP3 + 100 µm BA
26%
HE
DiOC6

Figure 10 D

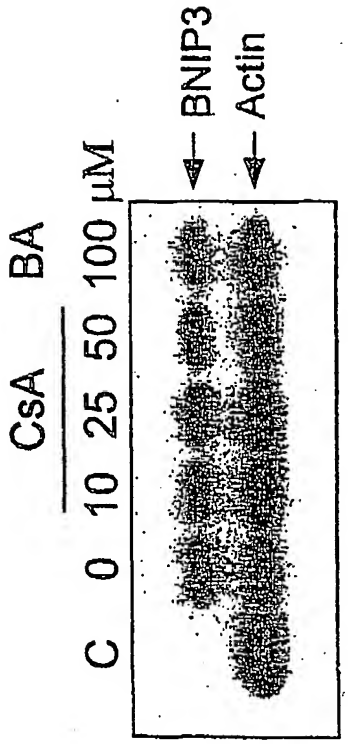


Figure 10E

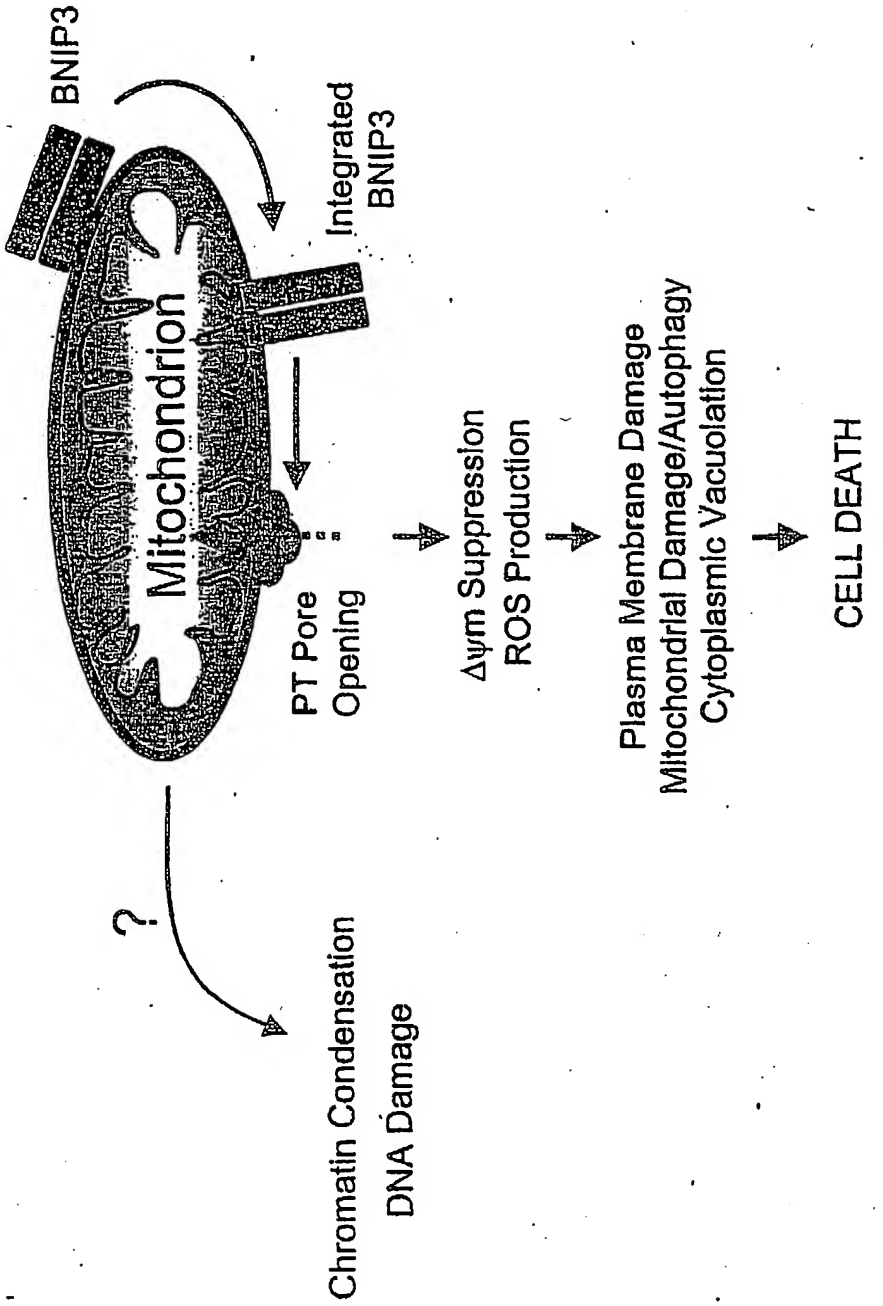

BNIP3
Integrated BNIP3
Mitochondrion
PT Pore Opening
Δψm Suppression
ROS Production
Plasma Membrane Damage
Mitochondrial Damage/Autophagy
Cytoplasmic Vacuolation
CELL DEATH
?
Chromatin Condensation
DNA Damage

Figure 11

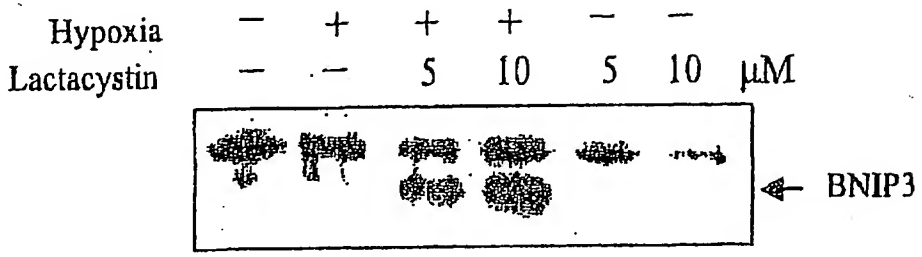


Figure 1: Neonatal cardiac myocytes were incubated in hypoxic (+) or normoxic (-) conditions for 24 hours. Cell were treated with lactacystin at the indicated concentrations and lysates harvested for western blotting with anti-BNip3 antibody. BNip3 band is indicated with an arrow. Lactacystin slows the degradation of the BNip3 protein and allows its detection by western blot.

Figure 12

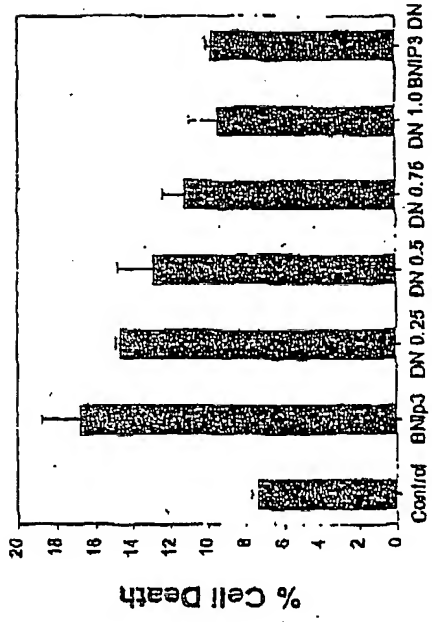
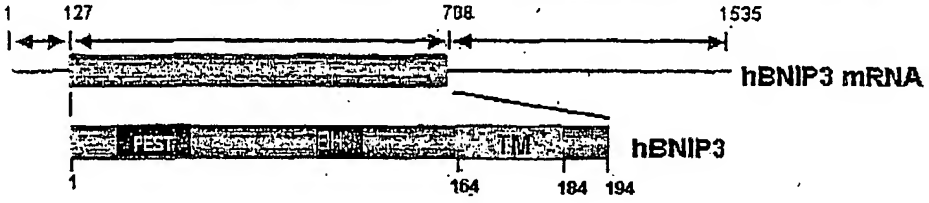


Figure 2: BNip3 induced cell death is inhibited by BNip3 DN (DN). Increasing concentrations of DN plasmid (0.25 to 1.0 µg) were added to a constant amount of BNip3 (1.0 µg). Cell death was determined by exclusion of propidium iodide. BNip3 DN was able to suppress the cell death induced by BNip3 to background levels at a 1.0 to1.0 µg plasmid ratio. The DN mutant of BNip3 contains amino acids 1-163, which is a deletion of the C-terminus of the protein that contains the transmembrane domain.

Figure 13

hBNIP3: Structure and functional domains



Functional Domains	hBNIP3
GrsB interacting site	E12-G41
GrsB cleavage site	D49
Bcl-2 binding domain	M1-D49
PEST domain	D42-D83
BH3 like domain	K104-D119
BNIP3 family homology domain	K112-K130
Transmembrane domain	V164-G184
Dimerization domain	V164-G188
Death associated domain	V164-F194

Figure 14